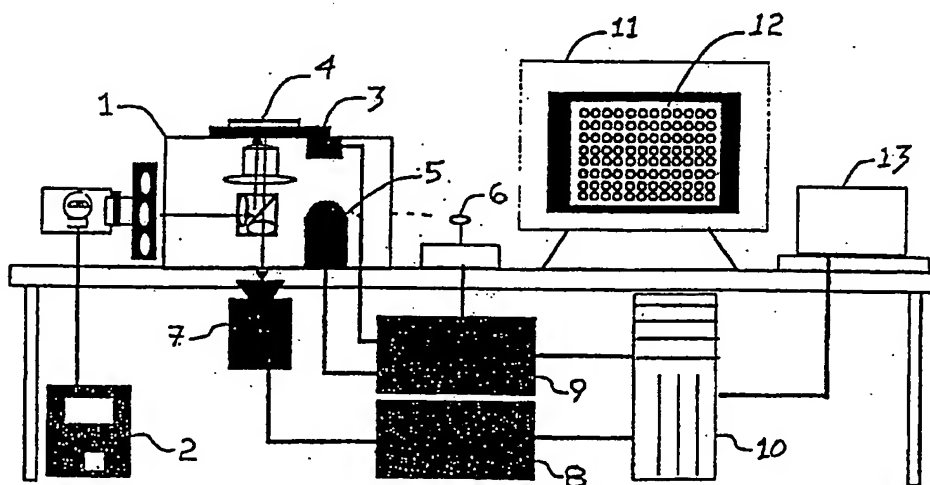




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US00/04794 <b>(22) International Filing Date:</b> 25 February 2000 (25.02.00) <b>(30) Priority Data:</b> 60/122,152 26 February 1999 (26.02.99) US 60/123,399 8 March 1999 (08.03.99) US 09/352,171 12 July 1999 (12.07.99) US <b>(71) Applicant (for all designated States except US):</b> CELLOMICS, INC. [US/US]; 635 William Pitt Way, Pittsburgh, PA 15238 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> GIULIANO, Kenneth, A. [US/US]; 351 Hawthorne Road, Pittsburgh, PA 15209 (US). KAPUR, Ravi [US/US]; 2942 E. Bardoneer Road, Gibsonia, PA 15044 (US). <b>(74) Agent:</b> HARPER, David, S.; McDonnell, Boehnen, Hulbert & Berghoff, Suite 3200, 300 South Wacker Drive, Chicago, IL 60606 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>

(54) Title: A SYSTEM FOR CELL-BASED SCREENING



## (57) Abstract

The present invention provides systems, methods, screens, reagents and kits for optical system analysis of cells to rapidly determine the distribution, environment, or activity of fluorescently labeled reporter molecules in cells for the purpose of screening large numbers of compounds for those that specifically affect particular biological functions.

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## A SYSTEM FOR CELL-BASED SCREENING

### 5 Cross Reference

This application claims priority to U.S. Provisional Applications for Patent Serial Nos. 60/122,152 (February 26, 1999), 60/123,399 (March 8, 1999), 09/352,141, (July 12, 1999), 60/151,797 (August 31, 1999), 60/168,408 (December 1, 1999); and is a continuation in part of 09/430,656 (October 29, 1999); 09/398,965 filed September 10 17, 1999 which is a continuation in part of Serial No. 09/031,271 filed February 27, 1998 which is a continuation in part of U.S. Application S/N 08/810983, filed on February 27, 1997.

### 15 Field of The Invention

This invention is in the field of fluorescence-based cell and molecular biochemical assays for drug discovery.

### 20 Background of the Invention

Drug discovery, as currently practiced in the art, is a long, multiple step process involving identification of specific disease targets, development of an assay based on a specific target, validation of the assay, optimization and automation of the assay to produce a screen, high throughput screening of compound libraries using the assay to 25 identify "hits", hit validation and hit compound optimization. The output of this process is a lead compound that goes into pre-clinical and, if validated, eventually into clinical trials. In this process, the screening phase is distinct from the assay development phases, and involves testing compound efficacy in living biological systems.

30 Historically, drug discovery is a slow and costly process, spanning numerous years and consuming hundreds of millions of dollars per drug created. Developments in the areas of genomics and high throughput screening have resulted in increased capacity and efficiency in the areas of target identification and volume of compounds

Historically, drug discovery is a slow and costly process, spanning numerous years and consuming hundreds of millions of dollars per drug created. Developments in the areas of genomics and high throughput screening have resulted in increased capacity and efficiency in the areas of target identification and volume of compounds screened. Significant advances in automated DNA sequencing, PCR application, positional cloning, hybridization arrays, and bioinformatics have greatly increased the number of genes (and gene fragments) encoding potential drug screening targets. However, the basic scheme for drug screening remains the same.

Validation of genomic targets as points for therapeutic intervention using the existing methods and protocols has become a bottleneck in the drug discovery process due to the slow, manual methods employed, such as *in vivo* functional models, functional analysis of recombinant proteins, and stable cell line expression of candidate genes. Primary DNA sequence data acquired through automated sequencing does not permit identification of gene function, but can provide information about common "motifs" and specific gene homology when compared to known sequence databases. Genomic methods such as subtraction hybridization and RADE (rapid amplification of differential expression) can be used to identify genes that are up or down regulated in a disease state model. However, identification and validation still proceed down the same pathway. Some proteomic methods use protein identification (global expression arrays, 2D electrophoresis, combinatorial libraries) in combination with reverse genetics to identify candidate genes of interest. Such putative "disease associated sequences" or DAS isolated as intact cDNA are a great advantage to these methods, but they are identified by the hundreds without providing any information regarding type, activity, and distribution of the encoded protein. Choosing a subset of DAS as drug screening targets is "random", and thus extremely inefficient, without functional data to provide a mechanistic link with disease. It is necessary, therefore, to provide new technologies to rapidly screen DAS to establish biological function, thereby improving target validation and candidate optimization in drug discovery.

There are three major avenues for improving early drug discovery productivity. First, there is a need for tools that provide increased information handling capability. Bioinformatics has blossomed with the rapid development of DNA sequencing systems and the evolution of the genomics database. Genomics is beginning to play a critical

role in the identification of potential new targets. Proteomics has become indispensable in relating structure and function of protein targets in order to predict drug interactions. However, the next level of biological complexity is the cell. Therefore, there is a need to acquire, manage and search multi-dimensional information from cells. Secondly, there is a need for higher throughput tools. Automation is a key to improving productivity as has already been demonstrated in DNA sequencing and high throughput primary screening. The instant invention provides for automated systems that extract multiple parameter information from cells that meet the need for higher throughput tools. The instant invention also provides for miniaturizing the methods, thereby allowing increased throughput, while decreasing the volumes of reagents and test compounds required in each assay.

Radioactivity has been the dominant read-out in early drug discovery assays. However, the need for more information, higher throughput and miniaturization has caused a shift towards using fluorescence detection. Fluorescence-based reagents can yield more powerful, multiple parameter assays that are higher in throughput and information content and require lower volumes of reagents and test compounds. Fluorescence is also safer and less expensive than radioactivity-based methods.

Screening of cells treated with dyes and fluorescent reagents is well known in the art. There is a considerable body of literature related to genetic engineering of cells to produce fluorescent proteins, such as modified green fluorescent protein (GFP), as a reporter molecule. Some properties of wild-type GFP are disclosed by Morise et al. (*Biochemistry* 13 (1974), p. 2656-2662), and Ward et al. (*Photochem. Photobiol.* 31 (1980), p. 611-615). The GFP of the jellyfish *Aequorea victoria* has an excitation maximum at 395 nm and an emission maximum at 510 nm, and does not require an exogenous factor for fluorescence activity. Uses for GFP disclosed in the literature are widespread and include the study of gene expression and protein localization (Chalfie et al., *Science* 263 (1994), p. 12501-12504), as a tool for visualizing subcellular organelles (Rizzuto et al., *Curr. Biology* 5 (1995), p. 635-642), visualization of protein transport along the secretory pathway (Kaether and Gerdes, *FEBS Letters* 369 (1995), p. 267-271), expression in plant cells (Hu and Cheng, *FEBS Letters* 369 (1995), p. 331-334) and *Drosophila* embryos (Davis et al., *Dev. Biology* 170 (1995), p. 726-729), and as a reporter molecule fused to another protein of interest (U. S. Patent

5,491,084). Similarly, WO96/23898 relates to methods of detecting biologically active substances affecting intracellular processes by utilizing a GFP construct having a protein kinase activation site. This patent, and all other patents referenced in this application are incorporated by reference in their entirety.

5 Numerous references are related to GFP proteins in biological systems. For example, WO 96/09598 describes a system for isolating cells of interest utilizing the expression of a GFP like protein. WO 96/27675 describes the expression of GFP in plants. WO 95/21191 describes modified GFP protein expressed in transformed organisms to detect mutagenesis. U. S. Patents 5,401,629 and 5,436,128 describe  
10 assays and compositions for detecting and evaluating the intracellular transduction of an extracellular signal using recombinant cells that express cell surface receptors and contain reporter gene constructs that include transcriptional regulatory elements that are responsive to the activity of cell surface receptors.

Performing a screen on many thousands of compounds requires parallel  
15 handling and processing of many compounds and assay component reagents. Standard high throughput screens ("HTS") use mixtures of compounds and biological reagents along with some indicator compound loaded into arrays of wells in standard microtiter plates with 96 or 384 wells. The signal measured from each well, either fluorescence emission, optical density, or radioactivity, integrates the signal from all the material in  
20 the well giving an overall population average of all the molecules in the well.

Science Applications International Corporation (SAIC) 130 Fifth Avenue, Seattle, WA. 98109) describes an imaging plate reader. This system uses a CCD camera to image the whole area of a 96 well plate. The image is analyzed to calculate the total fluorescence per well for all the material in the well.

25 Molecular Devices, Inc. (Sunnyvale, CA) describes a system (FLIPR) which uses low angle laser scanning illumination and a mask to selectively excite fluorescence within approximately 200 microns of the bottoms of the wells in standard 96 well plates in order to reduce background when imaging cell monolayers. This system uses a CCD camera to image the whole area of the plate bottom. Although this system  
30 measures signals originating from a cell monolayer at the bottom of the well, the signal measured is averaged over the area of the well and is therefore still considered a measurement of the average response of a population of cells. The image is analyzed to

calculate the total fluorescence per well for cell-based assays. Fluid delivery devices have also been incorporated into cell based screening systems, such as the FLIPR system, in order to initiate a response, which is then observed as a whole well population average response using a macro-imaging system.

5        In contrast to high throughput screens, various high-content screens ("HCS") have been developed to address the need for more detailed information about the temporal-spatial dynamics of cell constituents and processes. High-content screens automate the extraction of multicolor fluorescence information derived from specific fluorescence-based reagents incorporated into cells (Giuliano and Taylor (1995), *Curr.*  
10 *Op. Cell Biol.* 7:4; Giuliano et al. (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405). Cells are analyzed using an optical system that can measure spatial, as well as temporal dynamics. (Farkas et al. (1993) *Ann. Rev. Physiol.* 55:785; Giuliano et al. (1990) In *Optical Microscopy for Biology*. B. Herman and K. Jacobson (eds.), pp. 543-557. Wiley-Liss, New York; Hahn et al (1992) *Nature* 359:736; Waggoner et al. (1996)  
15 *Hum. Pathol.* 27:494). The concept is to treat each cell as a "well" that has spatial and temporal information on the activities of the labeled constituents.

The types of biochemical and molecular information now accessible through fluorescence-based reagents applied to cells include ion concentrations, membrane potential, specific translocations, enzyme activities, gene expression, as well as the  
20 presence, amounts and patterns of metabolites, proteins, lipids, carbohydrates, and nucleic acid sequences (DeBiasio et al., (1996) *Mol. Biol. Cell.* 7:1259; Giuliano et al., (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405; Heim and Tsien, (1996) *Curr. Biol.* 6:178).

High-content screens can be performed on either fixed cells, using fluorescently  
25 labeled antibodies, biological ligands, and/or nucleic acid hybridization probes, or live cells using multicolor fluorescent indicators and "biosensors." The choice of fixed or live cell screens depends on the specific cell-based assay required.

Fixed cell assays are the simplest, since an array of initially living cells in a microtiter plate format can be treated with various compounds and doses being tested,  
30 then the cells can be fixed, labeled with specific reagents, and measured. No environmental control of the cells is required after fixation. Spatial information is acquired, but only at one time point. The availability of thousands of antibodies,

ligands and nucleic acid hybridization probes that can be applied to cells makes this an attractive approach for many types of cell-based screens. The fixation and labeling steps can be automated, allowing efficient processing of assays.

Live cell assays are more sophisticated and powerful, since an array of living  
5 cells containing the desired reagents can be screened over time, as well as space. Environmental control of the cells (temperature, humidity, and carbon dioxide) is required during measurement, since the physiological health of the cells must be maintained for multiple fluorescence measurements over time. There is a growing list  
10 of fluorescent physiological indicators and "biosensors" that can report changes in biochemical and molecular activities within cells (Giuliano et al., (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405; Hahn et al., (1993) In *Fluorescent and Luminescent Probes for Biological Activity*. W.T. Mason, (ed.), pp. 349-359, Academic Press, San Diego).

The availability and use of fluorescence-based reagents has helped to advance  
15 the development of both fixed and live cell high-content screens. Advances in instrumentation to automatically extract multicolor, high-content information has recently made it possible to develop HCS into an automated tool. An article by Taylor, et al. (*American Scientist* 80 (1992), p. 322-335) describes many of these methods and their applications. For example, Proffitt et. al. (*Cytometry* 24: 204-213 (1996)) describe  
20 a semi-automated fluorescence digital imaging system for quantifying relative cell numbers in situ in a variety of tissue culture plate formats, especially 96-well microtiter plates. The system consists of an epifluorescence inverted microscope with a motorized stage, video camera, image intensifier, and a microcomputer with a PC-Vision digitizer. Turbo Pascal software controls the stage and scans the plate taking  
25 multiple images per well. The software calculates total fluorescence per well, provides for daily calibration, and configures easily for a variety of tissue culture plate formats. Thresholding of digital images and reagents which fluoresce only when taken up by living cells are used to reduce background fluorescence without removing excess fluorescent reagent.

30 Scanning confocal microscope imaging (Go et al., (1997) *Analytical Biochemistry* 247:210-215; Goldman et al., (1995) *Experimental Cell Research* 221:311-319) and multiphoton microscope imaging (Denk et al., (1990) *Science*

248:73; Gratton et al., (1994) *Proc. of the Microscopical Society of America*, pp. 154-155) are also well established methods for acquiring high resolution images of microscopic samples. The principle advantage of these optical systems is the very shallow depth of focus, which allows features of limited axial extent to be resolved against the background. For example, it is possible to resolve internal cytoplasmic features of adherent cells from the features on the cell surface. Because scanning multiphoton imaging requires very short duration pulsed laser systems to achieve the high photon flux required, fluorescence lifetimes can also be measured in these systems (Lakowicz et al., (1992) *Anal. Biochem.* 202:316-330; Gerritsen et al. (1997), *J. of Fluorescence* 7:11-15)), providing additional capability for different detection modes. Small, reliable and relatively inexpensive laser systems, such as laser diode pumped lasers, are now available to allow multiphoton confocal microscopy to be applied in a fairly routine fashion.

A combination of the biological heterogeneity of cells in populations (Bright, et al., (1989). *J. Cell. Physiol.* 141:410; Giuliano, (1996) *Cell Motil. Cytoskel.* 35:237)) as well as the high spatial and temporal frequency of chemical and molecular information present within cells, makes it impossible to extract high-content information from populations of cells using existing whole microtiter plate readers. No existing high-content screening platform has been designed for multicolor, fluorescence-based screens using cells that are analyzed individually. Similarly, no method is currently available that combines automated fluid delivery to arrays of cells for the purpose of systematically screening compounds for the ability to induce a cellular response that is identified by HCS analysis, especially from cells grown in microtiter plates. Furthermore, no method exists in the art combining high throughput well-by-well measurements to identify "hits" in one assay followed by a second high content cell-by-cell measurement on the same plate of only those wells identified as hits.

The instant invention provides systems, methods, and screens that combine high throughput screening (HTS) and high content screening (HCS) that significantly improve target validation and candidate optimization by combining many cell screening formats with fluorescence-based molecular reagents and computer-based feature extraction, data analysis, and automation, resulting in increased quantity and speed of

data collection, shortened cycle times, and, ultimately, faster evaluation of promising drug candidates. The instant invention also provides for miniaturizing the methods, thereby allowing increased throughput, while decreasing the volumes of reagents and test compounds required in each assay.

5

### SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a method for analyzing cells comprising providing cells containing fluorescent reporter molecules in an array of locations, treating the cells in the array of locations with one or more reagents, 10 imaging numerous cells in each location with fluorescence optics, converting the optical information into digital data, utilizing the digital data to determine the distribution, environment or activity of the fluorescently labeled reporter molecules in the cells and the distribution of the cells, and interpreting that information in terms of a positive, negative or null effect of the compound being tested on the biological 15 function

In this embodiment, the method rapidly determines the distribution, environment, or activity of fluorescently labeled reporter molecules in cells for the purpose of screening large numbers of compounds for those that specifically affect particular biological functions. The array of locations may be a microtiter plate or a 20 microchip which is a microplate having cells in an array of locations. In a preferred embodiment, the method includes computerized means for acquiring, processing, displaying and storing the data received. In a preferred embodiment, the method further comprises automated fluid delivery to the arrays of cells. In another preferred embodiment, the information obtained from high throughput measurements on the 25 same plate are used to selectively perform high content screening on only a subset of the cell locations on the plate.

In another aspect of the present invention, a cell screening system is provided that comprises:

- a high magnification fluorescence optical system having a microscope 30 objective,



- an XY stage adapted for holding a plate containing an array of cells and having a means for moving the plate for proper alignment and focusing on the cell arrays;
- a digital camera;
- 5     • a light source having optical means for directing excitation light to cell arrays and a means for directing fluorescent light emitted from the cells to the digital camera; and
- a computer means for receiving and processing digital data from the digital camera wherein the computer means includes a digital frame grabber for  
10     receiving the images from the camera, a display for user interaction and display of assay results, digital storage media for data storage and archiving, and a means for control, acquisition, processing and display of results.

15     In a preferred embodiment, the cell screening system further comprises a computer screen operatively associated with the computer for displaying data. In another preferred embodiment, the computer means for receiving and processing digital data from the digital camera stores the data in a bioinformatics data base. In a further preferred embodiment, the cell screening system further comprises a reader that measures a signal from many or all the wells in parallel. In another preferred  
20     embodiment, the cell screening system further comprises a mechanical-optical means for changing the magnification of the system, to allow changing modes between high throughput and high content screening. In another preferred embodiment, the cell screening system further comprises a chamber and control system to maintain the temperature, CO<sub>2</sub> concentration and humidity surrounding the plate at levels required to  
25     keep cells alive. In a further preferred embodiment, the cell screening system utilizes a confocal scanning illumination and detection system.

30     In another aspect of the present invention, a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute procedures for defining the distribution and activity of specific cellular constituents and processes is provided. In a preferred embodiment, the cell screening system comprises a high magnification fluorescence optical system with a stage adapted for holding cells and a means for moving the stage, a digital camera, a

light source for receiving and processing the digital data from the digital camera, and a computer means for receiving and processing the digital data from the digital camera. Preferred embodiments of the machine readable storage medium comprise programs consisting of a set of instructions for causing a cell screening system to execute the procedures set forth in Figures 9, 11, 12, 13, 14 or 15. Another preferred embodiment comprises a program consisting of a set of instructions for causing a cell screening system to execute procedures for detecting the distribution and activity of specific cellular constituents and processes. In most preferred embodiments, the cellular processes include, but are not limited to, nuclear translocation of a protein, cellular hypertrophy, apoptosis, and protease-induced translocation of a protein.

In another preferred embodiment, a variety of automated cell screening methods are provided, including screens to identify compounds that affect transcription factor activity, protein kinase activity, cell morphology, microtubule structure, apoptosis, receptor internalization, and protease-induced translocation of a protein.

In another aspect, the present invention provides recombinant nucleic acids encoding a protease biosensor, comprising:

a. a first nucleic acid sequence that encodes at least one detectable polypeptide signal;

b. a second nucleic acid sequence that encodes at least one protease recognition site, wherein the second nucleic acid sequence is operatively linked to the first nucleic acid sequence that encodes the at least one detectable polypeptide signal; and

c. a third nucleic acid sequence that encodes at least one reactant target sequence, wherein the third nucleic acid sequence is operatively linked to the second nucleic acid sequence that encodes the at least one protease recognition site.

The present invention also provides the recombinant expression vectors capable of expressing the recombinant nucleic acids encoding protease biosensors, as well as genetically modified host cells that are transfected with the expression vectors.

The invention further provides recombinant protease biosensors, comprising

- a. a first domain comprising at least one detectable polypeptide signal;  
b. a second domain comprising at least one protease recognition site; and  
c. a third domain comprising at least one reactant target sequence;

wherein the first domain and the third domain are separated by the second domain.

In a further aspect, the present invention involves assays and reagents for characterizing a sample for the presence of a toxin. The method comprises the use of  
5 detector, classifier, and identifier classes of toxin biosensors to provide for various levels of toxin characterization.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows a diagram of the components of the cell-based scanning system.

10 Figure 2 shows a schematic of the microscope subassembly.

Figure 3 shows the camera subassembly.

Figure 4 illustrates cell scanning system process.

Figure 5 illustrates a user interface showing major functions to guide the user.

15 Figure 6 is a block diagram of the two platform architecture of the Dual Mode System for Cell Based Screening in which one platform uses a telescope lens to read all wells of a microtiter plate and a second platform that uses a higher magnification lens to read individual cells in a well.

20 Figure 7 is a detail of an optical system for a single platform architecture of the Dual Mode System for Cell Based Screening that uses a moveable 'telescope' lens to read all wells of a microtiter plate and a moveable higher magnification lens to read individual cells in a well.

Figure 8 is an illustration of the fluid delivery system for acquiring kinetic data on the Cell Based Screening System.

Figure 9 is a flow chart of processing step for the cell-based scanning system.

25 Figure 10 A-J illustrates the strategy of the Nuclear Translocation Assay.

Figure 11 is a flow chart defining the processing steps in the Dual Mode System for Cell Based Screening combining high throughput and high content screening of microtiter plates.

30 Figure 12 is a flow chart defining the processing steps in the High Throughput mode of the System for Cell Based Screening.

Figure 13 is a flow chart defining the processing steps in the High Content mode of the System for Cell Based Screening.

**Figure 14** is a flow chart defining the processing steps required for acquiring kinetic data in the High Content mode of the System for Cell Based Screening.

**Figure 15** is a flow chart defining the processing steps performed within a well during the acquisition of kinetic data.

5 **Figure 16** is an example of data from a known inhibitor of translocation.

**Figure 17** is an example of data from a known stimulator of translocation.

**Figure 18** illustrates data presentation on a graphical display.

10 **Figure 19** is an illustration of the data from the High Throughput mode of the System for Cell Based Screening, an example of the data passed to the High Content mode, the data acquired in the high content mode, and the results of the analysis of that data.

**Figure 20** shows the measurement of a drug-induced cytoplasm to nuclear translocation.

**Figure 21** illustrates a graphical user interface of the measurement shown in Figure 20.

15 **Figure 22** illustrates a graphical user interface, with data presentation, of the measurement shown in Fig. 20.

**Figure 23** is a graph representing the kinetic data obtained from the measurements depicted in Fig. 20.

**Figure 24** details a high-content screen of drug-induced apoptosis.

20 **Figure 25.** Graphs depicting changes in morphology upon induction of apoptosis. Staurosporine (A) and paclitaxel (B) induce classic nuclear fragmentation in L929 cells. BHK cells exhibit concentration dependent changes in response to staurosporine (C), but a more classical response to paclitaxel (D). MCF-7 cells exhibit either nuclear condensation (E) or fragmentation (F) in response to staurosporine and paclitaxel, respectively. In all cases, cells were exposed to the compounds for 30 hours.

25 **Figure 26** illustrates the dose response of cells to staurosporine in terms of both nuclear size and nuclear perimeter convolution.

30 **Figure 27.** Graphs depicting induction of apoptosis by staurosporine and paclitaxel leading to changes in peri-nuclear f-actin content. (A, B) Both apoptotic stimulators induce dose-dependent increases in f-actin content in L929 cells. (C) In BHK cells, staurosporine induces a dose-dependent increase in f-actin, whereas paclitaxel (D) produces results that are more variable. (E) MCF-7 cells exhibit either a decrease or increase depending on the concentration of staurosporine. (F) Paclitaxel induced

changes in f-actin content were highly variable and not significant. Cells were exposed to the compounds for 30 hours.

**Figure 28.** Graphs depicting mitochondrial changes in response to induction of apoptosis. L929 (A,B) and BHK (C,D) cells responded to both staurosporine (A,C) and paclitaxel (B,D) with increases in mitochondrial mass. MCF-7 cells exhibit either a decrease in membrane potential (E, staurosporine) or an increase in mitochondrial mass (F, paclitaxel) depending on the stimulus. Cells were exposed to the compounds for 30 hours. 28G is a graph showing the simultaneous measurement of staurosporine effects on mitochondrial mass and mitochondrial potential in BHK cells.

**Figure 29** shows the nucleic acid and amino acid sequence for various types of protease biosensor domains. (A) Signal sequences. (B) Protease recognition sites. (C) Product/Reactant target sequences

**Figure 30** shows schematically shows some basic organization of domains in the protease biosensors of the invention.

**Figure 31** is a schematic diagram of a specific 3-domain protease biosensor.

**Figure 32** is a photograph showing the effect of stimulation of apoptosis by cis-platin on BHK cells transfected with an expression vector that expresses the caspase biosensor shown in Figure 32.

**Figure 33** is a schematic diagram of a specific 4-domain protease biosensor.

**Figure 34** is a schematic diagram of a specific 4-domain protease biosensor, containing a nucleolar localization signal.

**Figure 35** is a schematic diagram of a specific 5-domain protease biosensor.

**Figure 36** shows the differential response in a dual labeling assay of the p38 MAPK and NF- $\kappa$ B pathways across three model toxins and two different cell types.

Treatments marked with an asterisk are different from controls at a 99% confidence level ( $p < 0.01$ ).

## **DETAILED DESCRIPTION OF THE INVENTION**

All cited patents, patent applications and other references are hereby incorporated by reference in their entirety.

As used herein, the following terms have the specified meaning:

*Markers of cellular domains.* Luminescent probes that have high affinity for specific cellular constituents including specific organelles or molecules. These probes can either be small luminescent molecules or fluorescently tagged macromolecules used as "labeling reagents", "environmental indicators", or "biosensors."

5       *Labeling reagents.* Labeling reagents include, but are not limited to, luminescently labeled macromolecules including fluorescent protein analogs and biosensors, luminescent macromolecular chimeras including those formed with the green fluorescent protein and mutants thereof, luminescently labeled primary or secondary antibodies that react with cellular antigens involved in a physiological  
10 response, luminescent stains, dyes, and other small molecules.

*Markers of cellular translocations.* Luminescently tagged macromolecules or organelles that move from one cell domain to another during some cellular process or physiological response. Translocation markers can either simply report location relative to the markers of cellular domains or they can also be "biosensors" that report  
15 some biochemical or molecular activity as well.

*Biosensors.* Macromolecules consisting of a biological functional domain and a luminescent probe or probes that report the environmental changes that occur either internally or on their surface. A class of luminescently labeled macromolecules designed to sense and report these changes have been termed "fluorescent-protein  
20 biosensors". The protein component of the biosensor provides a highly evolved molecular recognition moiety. A fluorescent molecule attached to the protein component in the proximity of an active site transduces environmental changes into fluorescence signals that are detected using a system with an appropriate temporal and spatial resolution such as the cell scanning system of the present invention. Because  
25 the modulation of native protein activity within the living cell is reversible, and because fluorescent-protein biosensors can be designed to sense reversible changes in protein activity, these biosensors are essentially reusable.

*Disease associated sequences ("DAS").* This term refers to nucleic acid sequences identified by standard techniques, such as primary DNA sequence data,  
30 genomic methods such as subtraction hybridization and RADE, and proteomic methods in combination with reverse genetics, as being of drug candidate compounds. The term does not mean that the sequence is only associated with a disease state.

High content screening (HCS) can be used to measure the effects of drugs on complex molecular events such as signal transduction pathways, as well as cell functions including, but not limited to, apoptosis, cell division, cell adhesion, locomotion, exocytosis, and cell-cell communication. Multicolor fluorescence permits multiple targets and cell processes to be assayed in a single screen. Cross-correlation of cellular responses will yield a wealth of information required for target validation and lead optimization.

In one aspect of the present invention, a cell screening system is provided comprising a high magnification fluorescence optical system having a microscope objective, an XY stage adapted for holding a plate with an array of locations for holding cells and having a means for moving the plate to align the locations with the microscope objective and a means for moving the plate in the direction to effect focusing; a digital camera; a light source having optical means for directing excitation light to cells in the array of locations and a means for directing fluorescent light emitted from the cells to the digital camera; and a computer means for receiving and processing digital data from the digital camera wherein the computer means includes: a digital frame grabber for receiving the images from the camera, a display for user interaction and display of assay results, digital storage media for data storage and archiving, and means for control, acquisition, processing and display of results.

Figure 1 is a schematic diagram of a preferred embodiment of the cell scanning system. An inverted fluorescence microscope is used 1, such as a Zeiss Axiovert inverted fluorescence microscope which uses standard objectives with magnification of 1-100x to the camera, and a white light source (e.g. 100W mercury-arc lamp or 75W xenon lamp) with power supply 2. There is an XY stage 3 to move the plate 4 in the XY direction over the microscope objective. A Z-axis focus drive 5 moves the objective in the Z direction for focusing. A joystick 6 provides for manual movement of the stage in the XYZ direction. A high resolution digital camera 7 acquires images from each well or location on the plate. There is a camera power supply 8, an automation controller 9 and a central processing unit 10. The PC 11 provides a display 12 and has associated software. The printer 13 provides for printing of a hard copy record.

Figure 2 is a schematic of one embodiment of the microscope assembly 1 of the invention, showing in more detail the XY stage 3, Z-axis focus drive 5, joystick 6, light source 2, and automation controller 9. Cables to the computer 15 and microscope 16, respectively, are provided. In addition, Figure 2 shows a 96 well microtiter plate 17 which is moved on the XY stage 3 in the XY direction. Light from the light source 2 passes through the PC controlled shutter 18 to a motorized filter wheel 19 with excitation filters 20. The light passes into filter cube 25 which has a dichroic mirror 26 and an emission filter 27. Excitation light reflects off the dichroic mirror to the wells in the microtiter plate 17 and fluorescent light 28 passes through the dichroic mirror 26 and the emission filter 27 and to the digital camera 7.

Figure 3 shows a schematic drawing of a preferred camera assembly. The digital camera 7, which contains an automatic shutter for exposure control and a power supply 31, receives fluorescent light 28 from the microscope assembly. A digital cable 30 transports digital signals to the computer.

The standard optical configurations described above use microscope optics to directly produce an enlarged image of the specimen on the camera sensor in order to capture a high resolution image of the specimen. This optical system is commonly referred to as 'wide field' microscopy. Those skilled in the art of microscopy will recognize that a high resolution image of the specimen can be created by a variety of other optical systems, including, but not limited to, standard scanning confocal detection of a focused point or line of illumination scanned over the specimen (Go et al. 1997, *supra*), and multi-photon scanning confocal microscopy (Denk et al., 1990, *supra*), both of which can form images on a CCD detector or by synchronous digitization of the analog output of a photomultiplier tube.

In screening applications, it is often necessary to use a particular cell line, or primary cell culture, to take advantage of particular features of those cells. Those skilled in the art of cell culture will recognize that some cell lines are contact inhibited, meaning that they will stop growing when they become surrounded by other cells, while other cell lines will continue to grow under those conditions and the cells will literally pile up, forming many layers. An example of such a cell line is the HEK 293 (ATCC CRL-1573) line. An optical system that can acquire images of single cell layers in multilayer preparations is required for use with cell lines that tend to form



layers. The large depth of field of wide field microscopes produces an image that is a projection through the many layers of cells, making analysis of subcellular spatial distributions extremely difficult in layer-forming cells. Alternatively, the very shallow depth of field that can be achieved on a confocal microscope, (about one micron),  
5 allows discrimination of a single cell layer at high resolution, simplifying the determination of the subcellular spatial distribution. Similarly, confocal imaging is preferable when detection modes such as fluorescence lifetime imaging are required.

The output of a standard confocal imaging attachment for a microscope is a digital image that can be converted to the same format as the images produced by the  
10 other cell screening system embodiments described above, and can therefore be processed in exactly the same way as those images. The overall control, acquisition and analysis in this embodiment is essentially the same. The optical configuration of the confocal microscope system, is essentially the same as that described above, except for the illuminator and detectors. Illumination and detection systems required for  
15 confocal microscopy have been designed as accessories to be attached to standard microscope optical systems such as that of the present invention (Zeiss, Germany). These alternative optical systems therefore can be easily integrated into the system as described above.

Figure 4 illustrates an alternative embodiment of the invention in which cell  
20 arrays are in microwells 40 on a microplate 41, described in co-pending U.S. Application S/N 08/865,341, incorporated by reference herein in its entirety. Typically the microplate is 20 mm by 30 mm as compared to a standard 96 well microtiter plate which is 86 mm by 129 mm. The higher density array of cells on a microplate allows the microplate to be imaged at a low resolution of a few microns per pixel for high  
25 throughput and particular locations on the microplate to be imaged at a higher resolution of less than 0.5 microns per pixel. These two resolution modes help to improve the overall throughput of the system.

The microplate chamber 42 serves as a microfluidic delivery system for the addition of compounds to cells. The microplate 41 in the microplate chamber 42 is  
30 placed in an XY microplate reader 43. Digital data is processed as described above. The small size of this microplate system increases throughput, minimizes reagent volume and allows control of the distribution and placement of cells for fast and precise

cell-based analysis. Processed data can be displayed on a PC screen 11 and made part of a bioinformatics data base 44. This data base not only permits storage and retrieval of data obtained through the methods of this invention, but also permits acquisition and storage of external data relating to cells. Figure 5 is a PC display which illustrates the operation of the software.

In an alternative embodiment, a high throughput system (HTS) is directly coupled with the HCS either on the same platform or on two separate platforms connected electronically (e.g. via a local area network). This embodiment of the invention, referred to as a dual mode optical system, has the advantage of increasing the throughput of a HCS by coupling it with a HTS and thereby requiring slower high resolution data acquisition and analysis only on the small subset of wells that show a response in the coupled HTS.

High throughput 'whole plate' reader systems are well known in the art and are commonly used as a component of an HTS system used to screen large numbers of compounds (Beggs (1997), *J. of Biomolec. Screening* 2:71-78; Macaffrey et al., (1996) *J. Biomolec. Screening* 1:187-190).

In one embodiment of dual mode cell based screening, a two platform architecture in which high throughput acquisition occurs on one platform and high content acquisition occurs on a second platform is provided (Figure 6). Processing occurs on each platform independently, with results passed over a network interface, or a single controller is used to process the data from both platforms.

As illustrated in Figure 6, an exemplified two platform dual mode optical system consists of two light optical instruments, a high throughput platform 60 and a high content platform 65, which read fluorescent signals emitted from cells cultured in microtiter plates or microwell arrays on a microplate, and communicate with each other via an electronic connection 64. The high throughput platform 60 analyzes all the wells in the whole plate either in parallel or rapid serial fashion. Those skilled in the art of screening will recognize that there are a many such commercially available high throughput reader systems that could be integrated into a dual mode cell based screening system (Topcount (Packard Instruments, Meriden, CT); Spectramax, Lumiskan (Molecular Devices, Sunnyvale, CA); Fluoroscan (Labsystems, Beverly, MA)). The high content platform 65, as described above, scans from well to well and

acquires and analyzes high resolution image data collected from individual cells within a well.

The HTS software, residing on the system's computer 62, controls the high throughput instrument, and results are displayed on the monitor 61. The HCS software, residing on it's computer system 67, controls the high content instrument hardware 65, optional devices (e.g. plate loader, environmental chamber, fluid dispenser), analyzes digital image data from the plate, displays results on the monitor 66 and manages data measured in an integrated database. The two systems can also share a single computer, in which case all data would be collected, processed and displayed on that computer, without the need for a local area network to transfer the data. Microtiter plates are transferred from the high throughput system to the high content system 63 either manually or by a robotic plate transfer device, as is well known in the art (Beggs (1997), *supra*; Mcaffrey (1996), *supra*).

In a preferred embodiment, the dual mode optical system utilizes a single platform system (Figure 7). It consists of two separate optical modules, an HCS module 203 and an HTS module 209 that can be independently or collectively moved so that only one at a time is used to collect data from the microtiter plate 201. The microtiter plate 201 is mounted in a motorized X,Y stage so it can be positioned for imaging in either HTS or HCS mode. After collecting and analyzing the HTS image data as described below, the HTS optical module 209 is moved out of the optical path and the HCS optical module 203 is moved into place.

The optical module for HTS 209 consists of a projection lens 214, excitation wavelength filter 213 and dichroic mirror 210 which are used to illuminate the whole bottom of the plate with a specific wavelength band from a conventional microscope lamp system (not illustrated). The fluorescence emission is collected through the dichroic mirror 210 and emission wavelength filter 211 by a lens 212 which forms an image on the camera 216 with sensor 215.

The optical module for HCS 203 consists of a projection lens 208, excitation wavelength filter 207 and dichroic mirror 204 which are used to illuminate the back aperture of the microscope objective 202, and thereby the field of that objective, from a standard microscope illumination system (not shown). The fluorescence emission is

collected by the microscope objective 202, passes through the dichroic mirror 204 and emission wavelength filter 205 and is focused by a tube lens 206 which forms an image on the same camera 216 with sensor 215.

In an alternative embodiment of the present invention, the cell screening system further comprises a fluid delivery device for use with the live cell embodiment of the method of cell screening (see below). Figure 8 exemplifies a fluid delivery device for use with the system of the invention. It consists of a bank of 12 syringe pumps 701 driven by a single motor drive. Each syringe 702 is sized according to the volume to be delivered to each well, typically between 1 and 100  $\mu$ L. Each syringe is attached via flexible tubing 703 to a similar bank of connectors which accept standard pipette tips 705. The bank of pipette tips are attached to a drive system so they can be lowered and raised relative to the microtiter plate 706 to deliver fluid to each well. The plate is mounted on an X,Y stage, allowing movement relative to the optical system 707 for data collection purposes. This set-up allows one set of pipette tips, or even a single pipette tip, to deliver reagent to all the wells on the plate. The bank of syringe pumps can be used to deliver fluid to 12 wells simultaneously, or to fewer wells by removing some of the tips.

In another aspect, the present invention provides a method for analyzing cells comprising providing an array of locations which contain multiple cells wherein the cells contain one or more fluorescent reporter molecules; scanning multiple cells in each of the locations containing cells to obtain fluorescent signals from the fluorescent reporter molecule in the cells; converting the fluorescent signals into digital data; and utilizing the digital data to determine the distribution, environment or activity of the fluorescent reporter molecule within the cells.

### *Cell Arrays*

Screening large numbers of compounds for activity with respect to a particular biological function requires preparing arrays of cells for parallel handling of cells and reagents. Standard 96 well microtiter plates which are 86 mm by 129 mm, with 6mm diameter wells on a 9mm pitch, are used for compatibility with current automated loading and robotic handling systems. The microplate is typically 20 mm by 30 mm, with cell locations that are 100-200 microns in dimension on a pitch of about 500

microns. Methods for making microplates are described in U.S. Patent Application Serial No. 08/865,341, incorporated by reference herein in its entirety. Microplates may consist of coplanar layers of materials to which cells adhere, patterned with materials to which cells will not adhere, or etched 3-dimensional surfaces of similarly patterned materials. For the purpose of the following discussion, the terms 'well' and 'microwell' refer to a location in an array of any construction to which cells adhere and within which the cells are imaged. Microplates may also include fluid delivery channels in the spaces between the wells. The smaller format of a microplate increases the overall efficiency of the system by minimizing the quantities of the reagents, storage and handling during preparation and the overall movement required for the scanning operation. In addition, the whole area of the microplate can be imaged more efficiently, allowing a second mode of operation for the microplate reader as described later in this document.

#### *Fluorescence Reporter Molecules*

A major component of the new drug discovery paradigm is a continually growing family of fluorescent and luminescent reagents that are used to measure the temporal and spatial distribution, content, and activity of intracellular ions, metabolites, macromolecules, and organelles. Classes of these reagents include labeling reagents that measure the distribution and amount of molecules in living and fixed cells, environmental indicators to report signal transduction events in time and space, and fluorescent protein biosensors to measure target molecular activities within living cells. A multiparameter approach that combines several reagents in a single cell is a powerful new tool for drug discovery.

The method of the present invention is based on the high affinity of fluorescent or luminescent molecules for specific cellular components. The affinity for specific components is governed by physical forces such as ionic interactions, covalent bonding (which includes chimeric fusion with protein-based chromophores, fluorophores, and lumiphores), as well as hydrophobic interactions, electrical potential, and, in some cases, simple entrapment within a cellular component. The luminescent probes can be small molecules, labeled macromolecules, or genetically engineered proteins, including, but not limited to green fluorescent protein chimeras.

Those skilled in this art will recognize a wide variety of fluorescent reporter molecules that can be used in the present invention, including, but not limited to, fluorescently labeled biomolecules such as proteins, phospholipids and DNA hybridizing probes. Similarly, fluorescent reagents specifically synthesized with particular chemical properties of binding or association have been used as fluorescent reporter molecules (Barak et al., (1997), *J. Biol. Chem.* 272:27497-27500; Southwick et al., (1990), *Cytometry* 11:418-430; Tsien (1989) in *Methods in Cell Biology*, Vol. 29 Taylor and Wang (eds.), pp. 127-156). Fluorescently labeled antibodies are particularly useful reporter molecules due to their high degree of specificity for attaching to a single molecular target in a mixture of molecules as complex as a cell or tissue.

The luminescent probes can be synthesized within the living cell or can be transported into the cell via several non-mechanical modes including diffusion, facilitated or active transport, signal-sequence-mediated transport, and endocytotic or pinocytotic uptake. Mechanical bulk loading methods, which are well known in the art, can also be used to load luminescent probes into living cells (Barber et al. (1996), *Neuroscience Letters* 207:17-20; Bright et al. (1996), *Cytometry* 24:226-233; McNeil (1989) in *Methods in Cell Biology*, Vol. 29, Taylor and Wang (eds.), pp. 153-173). These methods include electroporation and other mechanical methods such as scrape-loading, bead-loading, impact-loading, syringe-loading, hypertonic and hypotonic loading. Additionally, cells can be genetically engineered to express reporter molecules, such as GFP, coupled to a protein of interest as previously described (Chalfie and Prasher U.S. Patent No. 5,491,084; Cubitt et al. (1995), *Trends in Biochemical Science* 20:448-455).

Once in the cell, the luminescent probes accumulate at their target domain as a result of specific and high affinity interactions with the target domain or other modes of molecular targeting such as signal-sequence-mediated transport. Fluorescently labeled reporter molecules are useful for determining the location, amount and chemical environment of the reporter. For example, whether the reporter is in a lipophilic membrane environment or in a more aqueous environment can be determined (Giuliano et al. (1995), *Ann. Rev. of Biophysics and Biomolecular Structure* 24:405-434; Giuliano and Taylor (1995), *Methods in Neuroscience* 27:1-16). The pH environment of the reporter can be determined (Bright et al. (1989), *J. Cell Biology* 104:1019-1033;

Giuliano et al. (1987), *Anal. Biochem.* 167:362-371; Thomas et al. (1979), *Biochemistry* 18:2210-2218). It can be determined whether a reporter having a chelating group is bound to an ion, such as  $\text{Ca}^{++}$ , or not (Bright et al. (1989), In *Methods in Cell Biology*, Vol. 30, Taylor and Wang (eds.), pp. 157-192; Shimoura et al. (1988), *J. of Biochemistry* (Tokyo) 251:405-410; Tsien (1989) In *Methods in Cell Biology*, Vol. 30, Taylor and Wang (eds.), pp. 127-156).

Furthermore, certain cell types within an organism may contain components that can be specifically labeled that may not occur in other cell types. For example, epithelial cells often contain polarized membrane components. That is, these cells asymmetrically distribute macromolecules along their plasma membrane. Connective or supporting tissue cells often contain granules in which are trapped molecules specific to that cell type (e.g., heparin, histamine, serotonin, etc.). Most muscular tissue cells contain a sarcoplasmic reticulum, a specialized organelle whose function is to regulate the concentration of calcium ions within the cell cytoplasm. Many nervous tissue cells contain secretory granules and vesicles in which are trapped neurohormones or neurotransmitters. Therefore, fluorescent molecules can be designed to label not only specific components within specific cells, but also specific cells within a population of mixed cell types.

Those skilled in the art will recognize a wide variety of ways to measure fluorescence. For example, some fluorescent reporter molecules exhibit a change in excitation or emission spectra, some exhibit resonance energy transfer where one fluorescent reporter loses fluorescence, while a second gains in fluorescence, some exhibit a loss (quenching) or appearance of fluorescence, while some report rotational movements (Giuliano et al. (1995), *Ann. Rev. of Biophysics and Biomol. Structure* 24:405-434; Giuliano et al. (1995), *Methods in Neuroscience* 27:1-16).

#### *Scanning cell arrays*

Referring to Figure 9, a preferred embodiment is provided to analyze cells that comprises operator-directed parameters being selected based on the assay being conducted, data acquisition by the cell screening system on the distribution of fluorescent signals within a sample, and interactive data review and analysis. At the start of an automated scan the operator enters information 100 that describes the sample, specifies the filter settings and fluorescent channels to match the biological

labels being used and the information sought, and then adjusts the camera settings to match the sample brightness. For flexibility to handle a range of samples, the software allows selection of various parameter settings used to identify nuclei and cytoplasm, and selection of different fluorescent reagents, identification of cells of interest based on morphology or brightness, and cell numbers to be analyzed per well. These parameters are stored in the system's for easy retrieval for each automated run. The system's interactive cell identification mode simplifies the selection of morphological parameter limits such as the range of size, shape, and intensity of cells to be analyzed. The user specifies which wells of the plate the system will scan and how many fields or how many cells to analyze in each well. Depending on the setup mode selected by the user at step 101, the system either automatically pre-focuses the region of the plate to be scanned using an autofocus procedure to "find focus" of the plate 102 or the user interactively pre-focuses 103 the scanning region by selecting three "tag" points which define the rectangular area to be scanned. A least-squares fit "focal plane model" is then calculated from these tag points to estimate the focus of each well during an automated scan. The focus of each well is estimated by interpolating from the focal plane model during a scan.

During an automated scan, the software dynamically displays the scan status, including the number of cells analyzed, the current well being analyzed, images of each independent wavelength as they are acquired, and the result of the screen for each well as it is determined. The plate 4 (Figure 1) is scanned in a serpentine style as the software automatically moves the motorized microscope XY stage 3 from well to well and field to field within each well of a 96-well plate. Those skilled in the programming art will recognize how to adapt software for scanning of other microplate formats such as 24, 48, and 384 well plates. The scan pattern of the entire plate as well as the scan pattern of fields within each well are programmed. The system adjusts sample focus with an autofocus procedure 104 (Figure 9) through the Z axis focus drive 5, controls filter selection via a motorized filter wheel 19, and acquires and analyzes images of up to four different colors ("channels" or "wavelengths").

The autofocus procedure is called at a user selected frequency, typically for the first field in each well and then once every 4 to 5 fields within each well. The autofocus procedure calculates the starting Z-axis point by interpolating from the pre-calculated



plane focal model. Starting a programmable distance above or below this set point, the procedure moves the mechanical Z-axis through a number of different positions, acquires an image at each position, and finds the maximum of a calculated focus score that estimates the contrast of each image. The Z position of the image with the maximum focus score determines the best focus for a particular field. Those skilled in the art will recognize this as a variant of automatic focusing methods as described in Harms et al. in *Cytometry* 5 (1984), 236-243, Groen et al. in *Cytometry* 6 (1985), 81-91, and Firestone et al. in *Cytometry* 12 (1991), 195-206.

For image acquisition, the camera's exposure time is separately adjusted for each dye to ensure a high-quality image from each channel. Software procedures can be called, at the user's option, to correct for registration shifts between wavelengths by accounting for linear (X and Y) shifts between wavelengths before making any further measurements. The electronic shutter 18 is controlled so that sample photo-bleaching is kept to a minimum. Background shading and uneven illumination can be corrected by the software using methods known in the art (Bright et al. (1987), *J. Cell Biol.* 104:1019-1033).

In one channel, images are acquired of a primary marker 105 (Figure 9) (typically cell nuclei counterstained with DAPI or PI fluorescent dyes) which are segmented ("identified") using an adaptive thresholding procedure. The adaptive thresholding procedure 106 is used to dynamically select the threshold of an image for separating cells from the background. The staining of cells with fluorescent dyes can vary to an unknown degree across cells in a microtiter plate sample as well as within images of a field of cells within each well of a microtiter plate. This variation can occur as a result of sample preparation and/or the dynamic nature of cells. A global threshold is calculated for the complete image to separate the cells from background and account for field to field variation. These global adaptive techniques are variants of those described in the art. (Kittler et al. in *Computer Vision, Graphics, and Image Processing* 30 (1985), 125-147, Ridler et al. in *IEEE Trans. Systems, Man, and Cybernetics* (1978), 630-632.)

An alternative adaptive thresholding method utilizes local region thresholding in contrast to global image thresholding. Image analysis of local regions leads to better overall segmentation since staining of cell nuclei (as well as other labeled components)

can vary across an image. Using this global/local procedure, a reduced resolution image (reduced in size by a factor of 2 to 4) is first globally segmented (using adaptive thresholding) to find regions of interest in the image. These regions then serve as guides to more fully analyze the same regions at full resolution. A more localized  
 5 threshold is then calculated (again using adaptive thresholding) for each region of interest.

The output of the segmentation procedure is a binary image wherein the objects are white and the background is black. This binary image, also called a mask in the art, is used to determine if the field contains objects 107. The mask is labeled with a blob  
 10 labeling method whereby each object (or blob) has a unique number assigned to it. Morphological features, such as area and shape, of the blobs are used to differentiate blobs likely to be cells from those that are considered artifacts. The user pre-sets the morphological selection criteria by either typing in known cell morphological features or by using the interactive training utility. If objects of interest are found in the field,  
 15 images are acquired for all other active channels 108, otherwise the stage is advanced to the next field 109 in the current well. Each object of interest is located in the image for further analysis 110. The software determines if the object meets the criteria for a valid cell nucleus 111 by measuring its morphological features (size and shape). For each valid cell, the XYZ stage location is recorded, a small image of the cell is stored,  
 20 and features are measured 112.

The cell scanning method of the present invention can be used to perform many different assays on cellular samples by applying a number of analytical methods simultaneously to measure features at multiple wavelengths. An example of one such assay provides for the following measurements:

- 25 1. The total fluorescent intensity within the cell nucleus for colors 1-4
2. The area of the cell nucleus for color 1 (the primary marker)
3. The shape of the cell nucleus for color 1 is described by three shape features:
  - 30 a) perimeter squared area
  - b) box area ratio
  - c) height width ratio
4. The average fluorescent intensity within the cell nucleus for colors 1-4 (i.e. #1 divided by #2)
- 35 5. The total fluorescent intensity of a ring outside the nucleus (see Figure 10) that represents fluorescence of the cell's cytoplasm (cytoplasmic mask) for colors 2-4

6. The area of the cytoplasmic mask
7. The average fluorescent intensity of the cytoplasmic mask for colors 2-4 (i.e. #5 divided by #6)
8. The ratio of the average fluorescent intensity of the cytoplasmic mask to average fluorescent intensity within the cell nucleus for colors 2-4 (i.e. #7 divided by #4)
9. The difference of the average fluorescent intensity of the cytoplasmic mask and the average fluorescent intensity within the cell nucleus for colors 2-4 (i.e. #7 minus #4)
10. The number of fluorescent domains (also call spots, dots, or grains) within the cell nucleus for colors 2-4

Features 1 through 4 are general features of the different cell screening assays of the invention. These steps are commonly used in a variety of image analysis applications and are well known in art (Russ (1992) *The Image Processing Handbook*, CRC Press Inc.; Gonzales et al. (1987), *Digital Image Processing*. Addison-Wesley Publishing Co. pp. 391-448). Features 5-9 have been developed specifically to provide measurements of a cell's fluorescent molecules within the local cytoplasmic region of the cell and the translocation (i.e. movement) of fluorescent molecules from the cytoplasm to the nucleus. These features (steps 5-9) are used for analyzing cells in microplates for the inhibition of nuclear translocation. For example, inhibition of nuclear translocation of transcription factors provides a novel approach to screening intact cells (detailed examples of other types of screens will be provided below). A specific method measures the amount of probe in the nuclear region (feature 4) versus the local cytoplasmic region (feature 7) of each cell. Quantification of the difference between these two sub-cellular compartments provides a measure of cytoplasm-nuclear translocation (feature 9).

Feature 10 describes a screen used for counting of DNA or RNA probes within the nuclear region in colors 2-4. For example, probes are commercially available for identifying chromosome-specific DNA sequences (Life Technologies, Gaithersburg, MD; Genosys, Woodlands, TX; Biotechnologies, Inc., Richmond, CA; Bio 101, Inc., Vista, CA) Cells are three-dimensional in nature and when examined at a high magnification under a microscope one probe may be in-focus while another may be completely out-of-focus. The cell screening method of the present invention provides for detecting three-dimensional probes in nuclei by acquiring images from multiple focal planes. The software moves the Z-axis motor drive 5 (Figure 1) in small steps

where the step distance is user selected to account for a wide range of different nuclear diameters. At each of the focal steps, an image is acquired. The maximum gray-level intensity from each pixel in each image is found and stored in a resulting maximum projection image. The maximum projection image is then used to count the probes. The  
5 above method works well in counting probes that are not stacked directly above or below another one. To account for probes stacked on top of each other in the Z-direction, users can select an option to analyze probes in each of the focal planes acquired. In this mode, the scanning system performs the maximum plane projection method as discussed above, detects probe regions of interest in this image, then further  
10 analyzes these regions in all the focal plane images.

After measuring cell features 112 (Figure 9), the system checks if there are any unprocessed objects in the current field 113. If there are any unprocessed objects, it locates the next object 110 and determines whether it meets the criteria for a valid cell nucleus 111, and measures its features. Once all the objects in the current field are  
15 processed, the system determines whether analysis of the current plate is complete 114; if not, it determines the need to find more cells in the current well 115. If the need exists, the system advances the XYZ stage to the next field within the current well 109 or advances the stage to the next well 116 of the plate.

After a plate scan is complete, images and data can be reviewed with the  
20 system's image review, data review, and summary review facilities. All images, data, and settings from a scan are archived in the system's database for later review or for interfacing with a network information management system. Data can also be exported to other third-party statistical packages to tabulate results and generate other reports. Users can review the images alone of every cell analyzed by the system with an  
25 interactive image review procedure 117. The user can review data on a cell-by-cell basis using a combination of interactive graphs, a data spreadsheet of measured features, and images of all the fluorescence channels of a cell of interest with the interactive cell-by-cell data review procedure 118. Graphical plotting capabilities are provided in which data can be analyzed via interactive graphs such as histograms and  
30 scatter plots. Users can review summary data that are accumulated and summarized for all cells within each well of a plate with an interactive well-by-well data review

procedure 119. Hard copies of graphs and images can be printed on a wide range of standard printers.

As a final phase of a complete scan, reports can be generated on one or more statistics of the measured features. Users can generate a graphical report of data summarized on a well-by-well basis for the scanned region of the plate using an interactive report generation procedure 120. This report includes a summary of the statistics by well in tabular and graphical format and identification information on the sample. The report window allows the operator to enter comments about the scan for later retrieval. Multiple reports can be generated on many statistics and be printed with the touch of one button. Reports can be previewed for placement and data before being printed.

The above-recited embodiment of the method operates in a single high resolution mode referred to as the high content screening (HCS) mode. The HCS mode provides sufficient spatial resolution within a well (on the order of 1  $\mu\text{m}$ ) to define the distribution of material within the well, as well as within individual cells in the well. The high degree of information content accessible in that mode, comes at the expense of speed and complexity of the required signal processing.

In an alternative embodiment, a high throughput system (HTS) is directly coupled with the HCS either on the same platform or on two separate platforms connected electronically (e.g. via a local area network). This embodiment of the invention, referred to as a dual mode optical system, has the advantage of increasing the throughput of an HCS by coupling it with an HTS and thereby requiring slower high resolution data acquisition and analysis only on the small subset of wells that show a response in the coupled HTS.

High throughput 'whole plate' reader systems are well known in the art and are commonly used as a component of an HTS system used to screen large numbers of compounds (Beggs et al. (1997), *supra*; McCaffrey et al. (1996), *supra*). The HTS of the present invention is carried out on the microtiter plate or microwell array by reading many or all wells in the plate simultaneously with sufficient resolution to make determinations on a well-by-well basis. That is, calculations are made by averaging the total signal output of many or all the cells or the bulk of the material in each well.

Wells that exhibit some defined response in the HTS (the 'hits') are flagged by the system. Then on the same microtiter plate or microwell array, each well identified as a hit is measured via HCS as described above. Thus, the dual mode process involves:

1. Rapidly measuring numerous wells of a microtiter plate or microwell array,
- 5 2. Interpreting the data to determine the overall activity of fluorescently labeled reporter molecules in the cells on a well-by-well basis to identify "hits" (wells that exhibit a defined response),
3. Imaging numerous cells in each "hit" well, and
- 10 4. Interpreting the digital image data to determine the distribution, environment or activity of the fluorescently labeled reporter molecules in the individual cells (i.e. intracellular measurements) and the distribution of the cells to test for specific biological functions

In a preferred embodiment of dual mode processing (Figure 11), at the start of a  
15 run 301, the operator enters information 302 that describes the plate and its contents, specifies the filter settings and fluorescent channels to match the biological labels being used, the information sought and the camera settings to match the sample brightness. These parameters are stored in the system's database for easy retrieval for each automated run. The microtiter plate or microwell array is loaded into the cell screening  
20 system 303 either manually or automatically by controlling a robotic loading device. An optional environmental chamber 304 is controlled by the system to maintain the temperature, humidity and CO<sub>2</sub> levels in the air surrounding live cells in the microtiter plate or microwell array. An optional fluid delivery device 305 (see Figure 8) is controlled by the system to dispense fluids into the wells during the scan.

25 High throughput processing 306 is first performed on the microtiter plate or microwell array by acquiring and analyzing the signal from each of the wells in the plate. The processing performed in high throughput mode 307 is illustrated in Figure 12 and described below. Wells that exhibit some selected intensity response in this high throughput mode ("hits") are identified by the system. The system performs a  
30 conditional operation 308 that tests for hits. If hits are found, those specific hit wells are further analyzed in high content (micro level) mode 309. The processing performed in high content mode 312 is illustrated in Figure 13. The system then updates 310 the informatics database 311 with results of the measurements on the plate. If there are

more plates to be analyzed 313 the system loads the next plate 303; otherwise the analysis of the plates terminates 314.

The following discussion describes the high throughput mode illustrated in Figure 12. The preferred embodiment of the system, the single platform dual mode screening system, will be described. Those skilled in the art will recognize that operationally the dual platform system simply involves moving the plate between two optical systems rather than moving the optics. Once the system has been set up and the plate loaded, the system begins the HTS acquisition and analysis 401. The HTS optical module is selected by controlling a motorized optical positioning device 402 on the dual mode system. In one fluorescence channel, data from a primary marker on the plate is acquired 403 and wells are isolated from the plate background using a masking procedure 404. Images are also acquired in other fluorescence channels being used 405. The region in each image corresponding to each well 406 is measured 407. A feature calculated from the measurements for a particular well is compared with a predefined threshold or intensity response 408, and based on the result the well is either flagged as a "hit" 409 or not. The locations of the wells flagged as hits are recorded for subsequent high content mode processing. If there are wells remaining to be processed 410 the program loops back 406 until all the wells have been processed 411 and the system exits high throughput mode.

Following HTS analysis, the system starts the high content mode processing 501 defined in Figure 13. The system selects the HCS optical module 502 by controlling the motorized positioning system. For each "hit" well identified in high throughput mode, the XY stage location of the well is retrieved from memory or disk and the stage is then moved to the selected stage location 503. The autofocus procedure 504 is called for the first field in each hit well and then once every 5 to 8 fields within each well. In one channel, images are acquired of the primary marker 505 (typically cell nuclei counterstained with DAPI, Hoechst or PI fluorescent dye). The images are then segmented (separated into regions of nuclei and non-nuclei) using an adaptive thresholding procedure 506. The output of the segmentation procedure is a binary mask wherein the objects are white and the background is black. This binary image, also called a mask in the art, is used to determine if the field contains objects 507. The mask

is labeled with a blob labeling method whereby each object (or blob) has a unique number assigned to it. If objects are found in the field, images are acquired for all other active channels 508, otherwise the stage is advanced to the next field 514 in the current well. Each object is located in the image for further analysis 509. Morphological features, such as area and shape of the objects, are used to select objects likely to be cell nuclei 510, and discard (do no further processing on) those that are considered artifacts. For each valid cell nucleus, the XYZ stage location is recorded, a small image of the cell is stored, and assay specific features are measured 511. The system then performs multiple tests on the cells by applying several analytical methods to measure features at each of several wavelengths. After measuring the cell features, the systems checks if there are any unprocessed objects in the current field 512. If there are any unprocessed objects, it locates the next object 509 and determines whether it meets the criteria for a valid cell nucleus 510, and measures its features. After processing all the objects in the current field, the system determines whether it needs to find more cells or fields in the current well 513. If it needs to find more cells or fields in the current well it advances the XYZ stage to the next field within the current well 515. Otherwise, the system checks whether it has any remaining hit wells to measure 515. If so, it advances to the next hit well 503 and proceeds through another cycle of acquisition and analysis; otherwise the HCS mode is finished 516.

In an alternative embodiment of the present invention, a method of kinetic live cell screening is provided. The previously described embodiments of the invention are used to characterize the spatial distribution of cellular components at a specific point in time, the time of chemical fixation. As such, these embodiments have limited utility for implementing kinetic based screens, due to the sequential nature of the image acquisition, and the amount of time required to read all the wells on a plate. For example, since a plate can require 30 – 60 minutes to read through all the wells, only very slow kinetic processes can be measured by simply preparing a plate of live cells and then reading through all the wells more than once. Faster kinetic processes can be measured by taking multiple readings of each well before proceeding to the next well, but the elapsed time between the first and last well would be too long, and fast kinetic processes would likely be complete before reaching the last well.



The kinetic live cell extension of the invention enables the design and use of screens in which a biological process is characterized by its kinetics instead of, or in addition to, its spatial characteristics. In many cases, a response in live cells can be measured by adding a reagent to a specific well and making multiple measurements on that well with the appropriate timing. This dynamic live cell embodiment of the invention therefore includes apparatus for fluid delivery to individual wells of the system in order to deliver reagents to each well at a specific time in advance of reading the well. This embodiment thereby allows kinetic measurements to be made with temporal resolution of seconds to minutes on each well of the plate. To improve the overall efficiency of the dynamic live cell system, the acquisition control program is modified to allow repetitive data collection from sub-regions of the plate, allowing the system to read other wells between the time points required for an individual well.

Figure 8 describes an example of a fluid delivery device for use with the live cell embodiment of the invention and is described above. This set-up allows one set of pipette tips 705, or even a single pipette tip, to deliver reagent to all the wells on the plate. The bank of syringe pumps 701 can be used to deliver fluid to 12 wells simultaneously, or to fewer wells by removing some of the tips 705. The temporal resolution of the system can therefore be adjusted, without sacrificing data collection efficiency, by changing the number of tips and the scan pattern as follows. Typically, the data collection and analysis from a single well takes about 5 seconds. Moving from well to well and focusing in a well requires about 5 seconds, so the overall cycle time for a well is about 10 seconds. Therefore, if a single pipette tip is used to deliver fluid to a single well, and data is collected repetitively from that well, measurements can be made with about 5 seconds temporal resolution. If 6 pipette tips are used to deliver fluids to 6 wells simultaneously, and the system repetitively scans all 6 wells, each scan will require 60 seconds, thereby establishing the temporal resolution. For slower processes which only require data collection every 8 minutes, fluids can be delivered to one half of the plate, by moving the plate during the fluid delivery phase, and then repetitively scanning that half of the plate. Therefore, by adjusting the size of the sub-region being scanned on the plate, the temporal resolution can be adjusted without having to insert wait times between acquisitions. Because the system is continuously scanning and acquiring data, the overall time to collect a kinetic data set from the plate

is then simply the time to perform a single scan of the plate, multiplied by the number of time points required. Typically, 1 time point before addition of compounds and 2 or 3 time points following addition should be sufficient for screening purposes.

Figure 14 shows the acquisition sequence used for kinetic analysis. The start of processing 801 is configuration of the system, much of which is identical to the standard HCS configuration. In addition, the operator must enter information specific to the kinetic analysis being performed 802, such as the sub-region size, the number of time points required, and the required time increment. A sub-region is a group of wells that will be scanned repetitively in order to accumulate kinetic data. The size of the sub-region is adjusted so that the system can scan a whole sub-region once during a single time increment, thus minimizing wait times. The optimum sub-region size is calculated from the setup parameters, and adjusted if necessary by the operator. The system then moves the plate to the first sub-region 803, and to the first well in that sub-region 804 to acquire the prestimulation (time = 0) time points. The acquisition sequence performed in each well is exactly the same as that required for the specific HCS being run in kinetic mode. Figure 15 details a flow chart for that processing. All of the steps between the start 901 and the return 902 are identical to those described as steps 504 – 514 in Figure 13.

After processing each well in a sub-region, the system checks to see if all the wells in the sub-region have been processed 806 (Figure 14), and cycles through all the wells until the whole region has been processed. The system then moves the plate into position for fluid addition, and controls fluidic system delivery of fluids to the entire sub-region 807. This may require multiple additions for sub-regions which span several rows on the plate, with the system moving the plate on the X,Y stage between additions. Once the fluids have been added, the system moves to the first well in the sub-region 808 to begin acquisition of time points. The data is acquired from each well 809 and as before the system cycles through all the wells in the sub-region 810. After each pass through the sub-region, the system checks whether all the time points have been collected 811 and if not, pauses 813 if necessary 812 to stay synchronized with the requested time increment. Otherwise, the system checks for additional sub-regions on the plate 814 and either moves to the next sub-region 803 or finishes 815. Thus, the

kinetic analysis mode comprises operator identification of sub-regions of the microtiter plate or microwells to be screened, based on the kinetic response to be investigated, with data acquisitions within a sub-region prior to data acquisition in subsequent sub-regions.

## 5 *Specific Screens*

In another aspect of the present invention, cell screening methods and machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute procedures for defining the distribution and activity of specific cellular constituents and processes is provided. In a preferred  
10 embodiment, the cell screening system comprises a high magnification fluorescence optical system with a stage adapted for holding cells and a means for moving the stage, a digital camera, a light source for receiving and processing the digital data from the digital camera, and a computer means for receiving and processing the digital data from the digital camera. This aspect of the invention comprises programs that instruct the  
15 cell screening system to define the distribution and activity of specific cellular constituents and processes, using the luminescent probes, the optical imaging system, and the pattern recognition software of the invention. Preferred embodiments of the machine readable storage medium comprise programs consisting of a set of instructions for causing a cell screening system to execute the procedures set forth in Figures 9, 11,  
20 12, 13, 14 or 15. Another preferred embodiment comprises a program consisting of a set of instructions for causing a cell screening system to execute procedures for detecting the distribution and activity of specific cellular constituents and processes. In most preferred embodiments, the cellular processes include, but are not limited to, nuclear translocation of a protein, cellular morphology, apoptosis, receptor  
25 internalization, and protease-induced translocation of a protein.

In a preferred embodiment, the cell screening methods are used to identify compounds that modify the various cellular processes. The cells can be contacted with a test compound, and the effect of the test compound on a particular cellular process can be analyzed. Alternatively, the cells can be contacted with a test compound and a  
30 known agent that modifies the particular cellular process, to determine whether the test compound can inhibit or enhance the effect of the known agent. Thus, the methods can

be used to identify test compounds that increase or decrease a particular cellular response, as well as to identify test compounds that affects the ability of other agents to increase or decrease a particular cellular response.

5 In another preferred embodiment, the locations containing cells are analyzed using the above methods at low resolution in a high throughput mode, and only a subset of the locations containing cells are analyzed in a high content mode to obtain luminescent signals from the luminescently labeled reporter molecules in subcellular compartments of the cells being analyzed.

10 The following examples are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined in the claims appended hereto.

The various chemical compounds, reagents, dyes, and antibodies that are referred to in the following Examples are commercially available from such sources as Sigma Chemical (St. Louis, MO), Molecular Probes (Eugene, OR), Aldrich Chemical  
15 Company (Milwaukee, WI), Accurate Chemical Company (Westbury, NY), Jackson Immunolabs, and Clontech (Palo Alto, CA).

**Example 1 Cytoplasm to Nucleus Translocation Screening:**

**a. Transcription Factors**

20 Regulation of transcription of some genes involves activation of a transcription factor in the cytoplasm, resulting in that factor being transported into the nucleus where it can initiate transcription of a particular gene or genes. This change in transcription factor distribution is the basis of a screen for the cell-based screening system to detect compounds that inhibit or induce transcription of a particular gene or group of genes.  
25 A general description of the screen is given followed by a specific example.

The distribution of the transcription factor is determined by labeling the nuclei with a DNA specific fluorophore like Hoechst 33423 and the transcription factor with a specific fluorescent antibody. After autofocusing on the Hoechst labeled nuclei, an image of the nuclei is acquired in the cell-based screening system and used to create a  
30 mask by one of several optional thresholding methods, as described *supra*. The morphological descriptors of the regions defined by the mask are compared with the

user defined parameters and valid nuclear masks are identified and used with the following method to extract transcription factor distributions. Each valid nuclear mask is eroded to define a slightly smaller nuclear region. The original nuclear mask is then dilated in two steps to define a ring shaped region around the nucleus, which represents a cytoplasmic region. The average antibody fluorescence in each of these two regions is determined, and the difference between these averages is defined as the NucCyt Difference. Two examples of determining nuclear translocation are discussed below and illustrated in Figure 10A-J. Figure 10A illustrates an unstimulated cell with its nucleus 200 labeled with a blue fluorophore and a transcription factor in the cytoplasm 201 labeled with a green fluorophore. Figure 10B illustrates the nuclear mask 202 derived by the cell-based screening system. Figure 10C illustrates the cytoplasm 203 of the unstimulated cell imaged at a green wavelength. Figure 10D illustrates the nuclear mask 202 is eroded (reduced) once to define a nuclear sampling region 204 with minimal cytoplasmic distribution. The nucleus boundary 202 is dilated (expanded) several times to form a ring that is 2-3 pixels wide that is used to define the cytoplasmic sampling region 205 for the same cell. Figure 10E further illustrates a side view which shows the nuclear sampling region 204 and the cytoplasmic sampling region 205. Using these two sampling regions, data on nuclear translocation can be automatically analyzed by the cell-based screening system on a cell by cell basis. Figure 10F-J illustrates the strategy for determining nuclear translocation in a stimulated cell. Figure 10F illustrates a stimulated cell with its nucleus 206 labeled with a blue fluorophore and a transcription factor in the cytoplasm 207 labeled with a green fluorophore. The nuclear mask 208 in Figure 10G is derived by the cell based screening system. Figure 10H illustrates the cytoplasm 209 of a stimulated cell imaged at a green wavelength. Figure 10I illustrates the nuclear sampling region 211 and cytoplasmic sampling region 212 of the stimulated cell. Figure 10J further illustrates a side view which shows the nuclear sampling region 211 and the cytoplasmic sampling region 212.

A specific application of this method has been used to validate this method as a screen. A human cell line was plated in 96 well microtiter plates. Some rows of wells were titrated with IL-1, a known inducer of the NF-KB transcription factor. The cells were then fixed and stained by standard methods with a fluorescein labeled antibody to

the transcription factor, and Hoechst 33423. The cell-based screening system was used to acquire and analyze images from this plate and the NucCyt Difference was found to be strongly correlated with the amount of agonist added to the wells as illustrated in Figure 16. In a second experiment, an antagonist to the receptor for IL-1, IL-1RA was titrated in the presence of IL-1 $\alpha$ , progressively inhibiting the translocation induced by IL-1 $\alpha$ . The NucCyt Difference was found to strongly correlate with this inhibition of translocation, as illustrated in Figure 17.

Additional experiments have shown that the NucCyt Difference, as well as the NucCyt ratio, gives consistent results over a wide range of cell densities and reagent concentrations, and can therefore be routinely used to screen compound libraries for specific nuclear translocation activity. Furthermore, the same method can be used with antibodies to other transcription factors, or GFP-transcription factor chimeras, or fluorescently labeled transcription factors introduced into living or fixed cells, to screen for effects on the regulation of transcription factor activity.

Figure 18 is a representative display on a PC screen of data which was obtained in accordance with Example 1. Graph 1 180 plots the difference between the average antibody fluorescence in the nuclear sampling region and cytoplasmic sampling region, NucCyt Difference verses Well #. Graph 2 181 plots the average fluorescence of the antibody in the nuclear sampling region, NP1 average, versus the Well #. Graph 3 182 plots the average antibody fluorescence in the cytoplasmic sampling region, LIP1 average, versus Well #. The software permits displaying data from each cell. For example, Figure 18 shows a screen display 183, the nuclear image 184, and the fluorescent antibody image 185 for cell #26.

NucCyt Difference referred to in graph 1 180 of Figure 18 is the difference between the average cytoplasmic probe (fluorescent reporter molecule) intensity and the average nuclear probe (fluorescent reporter molecule) intensity. NP1 average referred to in graph 2 181 of Figure 18 is the average of cytoplasmic probe (fluorescent reporter molecule) intensity within the nuclear sampling region. LIP1 average referred to in graph 3 182 of Figure 18 is the average probe (fluorescent reporter molecule) intensity within the cytoplasmic sampling region.

It will be understood by one of skill in the art that this aspect of the invention can be performed using other transcription factors that translocate from the cytoplasm

to the nucleus upon activation. In another specific example, activation of the c-fos transcription factor was assessed by defining its spatial position within cells. Activated c-fos is found only within the nucleus, while inactivated c-fos resides within the cytoplasm.

5 3T3 cells were plated at 5000-10000 cells per well in a Polyfiltronics 96-well plate. The cells were allowed to attach and grow overnight. The cells were rinsed twice with 100  $\mu$ l serum-free medium, incubated for 24-30 hours in serum-free MEM culture medium, and then stimulated with platelet derived growth factor (PDGF-BB) (Sigma Chemical Co., St. Louis, MO) diluted directly into serum free medium at  
10 concentrations ranging from 1-50 ng/ml for an average time of 20 minutes.

Following stimulation, cells were fixed for 20 minutes in 3.7% formaldehyde solution in 1X Hanks buffered saline solution (HBSS). After fixation, the cells were washed with HBSS to remove residual fixative, permeabilized for 90 seconds with 0.5% Triton X-100 solution in HBSS, and washed twice with HBSS to remove residual  
15 detergent. The cells were then blocked for 15 minutes with a 0.1% solution of BSA in HBSS, and further washed with HBSS prior to addition of diluted primary antibody solution.

c-Fos rabbit polyclonal antibody (Calbiochem, PC05) was diluted 1:50 in HBSS, and 50  $\mu$ l of the dilution was applied to each well. Cells were incubated in the  
20 presence of primary antibody for one hour at room temperature, and then incubated for one hour at room temperature in a light tight container with goat anti-rabbit secondary antibody conjugated to ALEXA<sup>TM</sup> 488 (Molecular Probes), diluted 1:500 from a 100  $\mu$ g/ml stock in HBSS. Hoechst DNA dye (Molecular Probes) was then added at a 1:1000 dilution of the manufacturer's stock solution (10 mg/ml). The cells were then  
25 washed with HBSS, and the plate was sealed prior to analysis with the cell screening system of the invention. The data from these experiments demonstrated that the methods of the invention could be used to measure transcriptional activation of c-fos by defining its spatial position within cells.

One of skill in the art will recognize that while the following method is applied to  
30 detection of c-fos activation, it can be applied to the analysis of any transcription factor that translocates from the cytoplasm to the nucleus upon activation. Examples of such transcription factors include, but are not limited to fos and jun homologs, NF-KB

(nuclear factor kappa from B cells), NFAT (nuclear factor of activated T-lymphocytes), and STATs (signal transducer and activator of transcription) factors (For example, see Strehlow, I., and Schindler, C. 1998. *J. Biol. Chem.* 273:28049-28056; Chow, et al. 1997 *Science*. 278:1638-1641; Ding et al. 1998 *J. Biol. Chem.* 273:28897-28905; 5 Baldwin, 1996. *Annu Rev Immunol.* 14:649-83; Kuo, C.T., and J.M. Leiden. 1999. *Annu Rev Immunol.* 17:149-87; Rao, et al. 1997. *Annu Rev Immunol.* 15:707-47; Masuda, et al. 1998. *Cell Signal.* 10:599-611; Hoey, T., and U. Schindler. 1998. *Curr Opin Genet Dev.* 8:582-7; Liu, et al. 1998. *Curr Opin Immunol.* 10:271-8.)

Thus, in this aspect of the invention, indicator cells are treated with test 10 compounds and the distribution of luminescently labeled transcription factor is measured in space and time using a cell screening system, such as the one disclosed above. The luminescently labeled transcription factor may be expressed by or added to the cells either before, together with, or after contacting the cells with a test compound.

For example, the transcription factor may be expressed as a luminescently 15 labeled protein chimera by transfected indicator cells. Alternatively, the luminescently labeled transcription factor may be expressed, isolated, and bulk-loaded into the indicator cells as described above, or the transcription factor may be luminescently labeled after isolation. As a further alternative, the transcription factor is expressed by the indicator cell, which is subsequently contacted with a luminescent label, such as an 20 antibody, that detects the transcription factor.

In a further aspect, kits are provided for analyzing transcription factor activation, comprising an antibody that specifically recognizes a transcription factor of interest, and instructions for using the antibody for carrying out the methods described above. In a preferred embodiment, the transcription factor-specific antibody, or a secondary 25 antibody that detects the transcription factor antibody, is luminescently labeled. In further preferred embodiments, the kit contains cells that express the transcription factor of interest, and/or the kit contains a compound that is known to modify activation of the transcription factor of interest, including but not limited to platelet derived growth factor (PDGF) and serum, which both modify fos activation; and interleukin 30 1(IL-1) and tumor necrosis factor (TNF), which both modify NF-KB activation.

In another embodiment, the kit comprises a recombinant expression vector comprising a nucleic acid encoding a transcription factor of interest that translocates



from the cytoplasm to the nucleus upon activation, and instructions for using the expression vector to identify compounds that modify transcription factor activation in a cell of interest. Alternatively, the kits contain a purified, luminescently labeled transcription factor. In a preferred embodiment, the transcription factor is expressed as a fusion protein with a luminescent protein, including but not limited to green fluorescent protein, luciferase, or mutants or fragments thereof. In various preferred embodiments, the kit further contains cells that are transfected with the expression vector, an antibody or fragment that specifically bind to the transcription factor of interest, and/or a compound that is known to modify activation of the transcription factor of interest (as above).

*b. Protein Kinases*

The cytoplasm to nucleus screening methods can also be used to analyze the activation of any protein kinase that is present in an inactive state in the cytoplasm and is transported to the nucleus upon activation, or that phosphorylates a substrate that translocates from the cytoplasm to the nucleus upon phosphorylation. Examples of appropriate protein kinases include, but are not limited to extracellular signal-regulated protein kinases (ERKs), c-Jun amino-terminal kinases (JNKs), Fos regulating protein kinases (FRKs), p38 mitogen activated protein kinase (p38MAPK), protein kinase A (PKA), and mitogen activated protein kinase kinases (MAPKKs). (For example, see Hall, et al. 1999. *J Biol Chem.* 274:376-83; Han, et al. 1995. *Biochim. Biophys. Acta.* 1265:224-227; Jaaro et al. 1997. *Proc. Natl. Acad. Sci. U.S.A.* 94:3742-3747; Taylor, et al. 1994. *J. Biol. Chem.* 269:308-318; Zhao, Q., and F. S. Lee. 1999. *J Biol Chem.* 274:8355-8; Paolillo et al. 1999. *J Biol Chem.* 274:6546-52; Coso et al. 1995. *Cell* 81:1137-1146; Tibbles, L.A., and J.R. Woodgett. 1999. *Cell Mol Life Sci.* 55:1230-54; Schaeffer, H.J., and M.J. Weber. 1999. *Mol Cell Biol.* 19:2435-44.)

Alternatively, protein kinase activity is assayed by monitoring translocation of a luminescently labeled protein kinase substrate from the cytoplasm to the nucleus after being phosphorylated by the protein kinase of interest. In this embodiment, the substrate is non-phosphorylated and cytoplasmic prior to phosphorylation, and is translocated to the nucleus upon phosphorylation by the protein kinase. There is no requirement that the protein kinase itself translocates from the cytoplasm to the nucleus

in this embodiment. Examples of such substrates (and the corresponding protein kinase) include, but are not limited to c-jun (JNK substrate); fos (FRK substrate), and p38 (p38 MAPK substrate).

Thus, in these embodiments, indicator cells are treated with test compounds and the distribution of luminescently labeled protein kinase or protein kinase substrate is measured in space and time using a cell screening system, such as the one disclosed above. The luminescently labeled protein kinase or protein kinase substrate may be expressed by or added to the cells either before, together with, or after contacting the cells with a test compound. For example, the protein kinase or protein kinase substrate may be expressed as a luminescently labeled protein chimera by transfected indicator cells. Alternatively, the luminescently labeled protein kinase or protein kinase substrate may be expressed, isolated, and bulk-loaded into the indicator cells as described above, or the protein kinase or protein kinase substrate may be luminescently labeled after isolation. As a further alternative, the protein kinase or protein kinase substrate is expressed by the indicator cell, which is subsequently contacted with a luminescent label, such as a labeled antibody, that detects the protein kinase or protein kinase substrate.

In a further embodiment, protein kinase activity is assayed by monitoring the phosphorylation state (ie: phosphorylated or not phosphorylated) of a protein kinase substrate. In this embodiment, there is no requirement that either the protein kinase or the protein kinase substrate translocate from the cytoplasm to the nucleus upon activation. In a preferred embodiment, phosphorylation state is monitored by contacting the cells with an antibody that binds only to the phosphorylated form of the protein kinase substrate of interest (For example, as disclosed in U.S. Patent No. 5,599,681).

In another preferred embodiment, a biosensor of phosphorylation is used. For example, a luminescently labeled protein or fragment thereof can be fused to a protein that has been engineered to contain (a) a phosphorylation site that is recognized by a protein kinase of interest; and (b) a nuclear localization signal that is unmasked by the phosphorylation. Such a biosensor will thus be translocated to the nucleus upon phosphorylation, and its translocation can be used as a measure of protein kinase activation.

In another aspect, kits are provided for analyzing protein kinase activation, comprising a primary antibody that specifically binds to a protein kinase, a protein kinase substrate, or a phosphorylated form of the protein kinase substrate of interest and instructions for using the primary antibody to identify compounds that modify protein kinase activation in a cell of interest. In a preferred embodiment, the primary antibody, or a secondary antibody that detects the primary antibody, is luminescently labeled. In other preferred embodiments, the kit further comprises cells that express the protein kinase of interest, and/or a compound that is known to modify activation of the protein kinase of interest, including but not limited to dibutyryl cAMP (modifies PKA), forskolin (PKA), and anisomycin (p38MAPK).

Alternatively, the kits comprise an expression vector encoding a protein kinase or a protein kinase substrate of interest that translocates from the cytoplasm to the nucleus upon activation and instructions for using the expression vector to identify compounds that modify protein kinase activation in a cell of interest. Alternatively, the kits contain a purified, luminescently labeled protein kinase or protein kinase substrate. In a preferred embodiment, the protein kinase or protein kinase substrate of interest is expressed as a fusion protein with a luminescent protein. In further preferred embodiments, the kit further comprises cells that are transfected with the expression vector, an antibody or fragment thereof that specifically binds to the protein kinase or protein kinase substrate of interest, and/or a compound that is known to modify activation of the protein kinase of interest. (as above)

In another aspect, the present invention comprises a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the methods disclosed for analyzing transcription factor or protein kinase activation, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a digital camera, a means for directing fluorescence or luminescence emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera.

*Example 2 Automated Screen for Compounds that Modify Cellular Morphology*

Changes in cell size are associated with a number of cellular conditions, such as hypertrophy, cell attachment and spreading, differentiation, growth and division, necrotic and programmed cell death, cell motility, morphogenesis, tube formation, and colony formation.

For example, cellular hypertrophy has been associated with a cascade of alterations in gene expression and can be characterized in cell culture by an alteration in cell size, that is clearly visible in adherent cells growing on a coverslip.

Cell size can also be measured to determine the attachment and spreading of adherent cells. Cell spreading is the result of selective binding of cell surface receptors to substrate ligands and subsequent activation of signaling pathways to the cytoskeleton. Cell attachment and spreading to substrate molecules is an important step for the metastasis of cancer cells, leukocyte activation during the inflammatory response, keratinocyte movement during wound healing, and endothelial cell movement during angiogenesis. Compounds that affect these surface receptors, signaling pathways, or the cytoskeleton will affect cell spreading and can be screened by measuring cell size.

Total cellular area can be monitored by labeling the entire cell body or the cell cytoplasm using cytoskeletal markers, cytosolic volume markers, or cell surface markers, in conjunction with a DNA label. Examples of such labels (many available from Molecular Probes (Eugene, Oregon) and Sigma Chemical Co. (St. Louis, Missouri)) include the following:

<b>CELL SIZE AND AREA MARKERS</b>
<b>Cytoskeletal Markers</b>
• ALEXA™ 488 phalloidin (Molecular Probes, Oregon)
• Tubulin-green fluorescent protein chimeras
• Cytokeratin-green fluorescent protein chimeras
• Antibodies to cytoskeletal proteins
<b>Cytosolic Volume Markers</b>
• Green fluorescent proteins
• Chloromethylfluorescein diacetate (CMFDA)
• Calcein green
• BCECF/AM ester
• Rhodamine dextran
<b>Cell Surface Markers for Lipid, Protein, or Oligosaccharide</b>
• Dihexadecyl tetramethylindocarbocyanine perchlorate (DiIC16) lipid dyes
• Triethylammonium propyl dibutylamino styryl pyridinium (FM 4-64, FM 1-43) lipid dyes
• MITOTRACKER™ Green FM
• Lectins to oligosaccharides such as fluorescein concanavalin A or wheat germ agglutinin
• SYPRO™ Red non-specific protein markers
• Antibodies to various surface proteins such as epidermal growth factor
• Biotin labeling of surface proteins followed by fluorescent streptavidin labeling

Protocols for cell staining with these various agents are well known to those skilled in the art. Cells are stained live or after fixation and the cell area can be measured. For example, live cells stained with DiIC16 have homogeneously labeled plasma membranes, and the projected cross-sectional area of the cell is uniformly discriminated from background by fluorescence intensity of the dye. Live cells stained with cytosolic stains such as CMFDA produce a fluorescence intensity that is proportional to cell thickness. Although cell labeling is dimmer in thin regions of the cell, total cell area can be discriminated from background. Fixed cells can be stained with cytoskeletal markers such as ALEXA™ 488 phalloidin that label polymerized actin. Phalloidin does not homogeneously stain the cytoplasm, but still permits discrimination of the total cell area from background.

#### 15 *Cellular hypertrophy*

A screen to analyze cellular hypertrophy is implemented using the following strategy. Primary rat myocytes can be cultured in 96 well plates, treated with various compounds and then fixed and labeled with a fluorescent marker for the cell membrane or cytoplasm, or cytoskeleton, such as an antibody to a cell surface marker or a

fluorescent marker for the cytoskeleton like rhodamine-phalloidin, in combination with a DNA label like Hoechst.

After focusing on the Hoechst labeled nuclei, two images are acquired, one of the Hoechst labeled nuclei and one of the fluorescent cytoplasm image. The nuclei are identified by thresholding to create a mask and then comparing the morphological descriptors of the mask with a set of user defined descriptor values. Each non-nucleus image (or "cytoplasmic image") is then processed separately. The original cytoplasm image can be thresholded, creating a cytoplasmic mask image. Local regions containing cells are defined around the nuclei. The limits of the cells in those regions are then defined by a local dynamic threshold operation on the same region in the fluorescent antibody image. A sequence of erosions and dilations is used to separate slightly touching cells and a second set of morphological descriptors is used to identify single cells. The area of the individual cells is tabulated in order to define the distribution of cell sizes for comparison with size data from normal and hypertrophic cells.

Responses from entire 96-well plates (measured as average cytoplasmic area/cell) were analyzed by the above methods, and the results demonstrated that the assay will perform the same on a well-to-well, plate-to-plate, and day-to-day basis (below a 15% cov for maximum signal). The data showed very good correlation for each day, and that there was no variability due to well position in the plate.

The following totals can be computed for the field. The aggregate whole nucleus area is the number of nonzero pixels in the nuclear mask. The average whole nucleus area is the aggregate whole nucleus area divided by the total number of nuclei. For each cytoplasm image several values can be computed. These are the total cytoplasmic area, which is the count of nonzero pixels in the cytoplasmic mask. The aggregate cytoplasm intensity is the sum of the intensities of all pixels in the cytoplasmic mask. The cytoplasmic area per nucleus is the total cytoplasmic area divided by the total nucleus count. The cytoplasmic intensity per nucleus is the aggregate cytoplasm intensity divided by the total nucleus count. The average cytoplasm intensity is the aggregate cytoplasm intensity divided by the cytoplasm area. The cytoplasm nucleus ratio is the total cytoplasm area divided by the total nucleus area.

Additionally, one or more fluorescent antibodies to other cellular proteins, such as the major muscle proteins actin or myosin, can be included. Images of these additional labeled proteins can be acquired and stored with the above images, for later review, to identify anomalies in the distribution and morphology of these proteins in hypertrophic cells. This example of a multi-parametric screen allows for simultaneous analysis of cellular hypertrophy and changes in actin or myosin distribution.

One of skill in the art will recognize that while the example analyzes myocyte hypertrophy, the methods can be applied to analyzing hypertrophy, or general morphological changes in any cell type.

#### *Cell morphology assays for prostate carcinoma*

Cell spreading is a measure of the response of cell surface receptors to substrate attachment ligands. Spreading is proportional to the ligand concentration or to the concentration of compounds that reduce receptor-ligand function. One example of selective cell-substrate attachment is prostate carcinoma cell adhesion to the extracellular matrix protein collagen. Prostate carcinoma cells metastasize to bone via selective adhesion to collagen.

Compounds that interfere with metastasis of prostate carcinoma cells were screened as follows. PC3 human prostate carcinoma cells were cultured in media with appropriate stimulants and are passaged to collagen coated 96 well plates. Ligand concentration can be varied or inhibitors of cell spreading can be added to the wells. Examples of compounds that can affect spreading are receptor antagonists such as integrin- or proteoglycan-blocking antibodies, signaling inhibitors including phosphatidyl inositol-3 kinase inhibitors, and cytoskeletal inhibitors such as cytochalasin D. After two hours, cells were fixed and stained with ALEXA<sup>TM</sup> 488 phalloidin (Molecular Probes) and Hoechst 33342 as per the protocol for cellular hypertrophy. The size of cells under these various conditions, as measured by cytoplasmic staining, can be distinguished above background levels. The number of cells per field is determined by measuring the number of nuclei stained with the Hoechst DNA dye. The area per cell is found by dividing the cytoplasmic area (phalloidin image) by the cell number (Hoechst image). The size of cells is proportional to the ligand-receptor function. Since the area is determined by ligand

concentration and by the resultant function of the cell, drug efficacy, as well as drug potency, can be determined by this cell-based assay. Other measurements can be made as discussed above for cellular hypertrophy.

The methods for analyzing cellular morphology can be used in a combined high throughput-high content screen. In one example, the high throughput mode scans the whole well for an increase in fluorescent phalloidin intensity. A threshold is set above which both nuclei (Hoechst) and cells (phalloidin) are measured in a high content mode. In another example, an environmental biosensor (examples include, but are not limited to, those biosensors that are sensitive to calcium and pH changes) is added to the cells, and the cells are contacted with a compound. The cells are scanned in a high throughput mode, and those wells that exceed a pre-determined threshold for luminescence of the biosensor are scanned in a high content mode.

In a further aspect, kits are provided for analyzing cellular morphology, comprising a luminescent compound that can be used to specifically label the cell cytoplasm, membrane, or cytoskeleton (such as those described above), and instructions for using the luminescent compound to identify test stimuli that induce or inhibit changes in cellular morphology according to the above methods. In a preferred embodiment, the kit further comprises a luminescent marker for cell nuclei. In a further preferred embodiment, the kit comprises at least one compound that is known to modify cellular morphology, including, but not limited to integrin- or proteoglycan-blocking antibodies, signaling inhibitors including phosphatidyl inositol-3 kinase inhibitors, and cytoskeletal inhibitors such as cytochalasin D.

In another aspect, the present invention comprises a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the disclosed methods for analyzing cellular morphology, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a digital camera, a means for directing fluorescence or luminescence emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera.

### Example 3 *Dual Mode High Throughput and High-Content Screen*

The following example is a screen for activation of a G-protein coupled receptor (GPCR) as detected by the translocation of the GPCR from the plasma membrane to a



proximal nuclear location. This example illustrates how a high throughput screen can be coupled with a high-content screen in the dual mode System for Cell Based Screening.

G-protein coupled receptors are a large class of 7 trans-membrane domain cell surface receptors. Ligands for these receptors stimulate a cascade of secondary signals in the cell, which may include, but are not limited to,  $\text{Ca}^{++}$  transients, cyclic AMP production, inositol triphosphate ( $\text{IP}_3$ ) production and phosphorylation. Each of these signals are rapid, occurring in a matter of seconds to minutes, but are also generic. For example, many different GPCRs produce a secondary  $\text{Ca}^{++}$  signal when activated. Stimulation of a GPCR also results in the transport of that GPCR from the cell surface membrane to an internal, proximal nuclear compartment. This internalization is a much more receptor-specific indicator of activation of a particular receptor than are the secondary signals described above.

Figure 19 illustrates a dual mode screen for activation of a GPCR. Cells carrying a stable chimera of the GPCR with a blue fluorescent protein (BFP) would be loaded with the acetoxymethylester form of Fluo-3, a cell permeable calcium indicator (green fluorescence) that is trapped in living cells by the hydrolysis of the esters. They would then be deposited into the wells of a microtiter plate 601. The wells would then be treated with an array of test compounds using a fluid delivery system, and a short sequence of Fluo-3 images of the whole microtiter plate would be acquired and analyzed for wells exhibiting a calcium response (i.e., high throughput mode). The images would appear like the illustration of the microtiter plate 601 in Figure 19. A small number of wells, such as wells C4 and E9 in the illustration, would fluoresce more brightly due to the  $\text{Ca}^{++}$  released upon stimulation of the receptors. The locations of wells containing compounds that induced a response 602, would then be transferred to the HCS program and the optics switched for detailed cell by cell analysis of the blue fluorescence for evidence of GPCR translocation to the perinuclear region. The bottom of Figure 19 illustrates the two possible outcomes of the analysis of the high resolution cell data. The camera images a sub-region 604 of the well area 603, producing images of the fluorescent cells 605. In well C4, the uniform distribution of the fluorescence in the cells indicates that the receptor has not internalized, implying that the  $\text{Ca}^{++}$  response

seen was the result of the stimulation of some other signalling system in the cell. The cells in well E9 606 on the other hand, clearly indicate a concentration of the receptor in the perinuclear region clearly indicating the full activation of the receptor. Because only a few hit wells have to be analyzed with high resolution, the overall throughput of the dual mode system can be quite high, comparable to the high throughput system alone.

#### *Example 4      Kinetic High Content Screen*

The following is an example of a screen to measure the kinetics of internalization of a receptor. As described above, the stimulation of a GPCR, results in the internalization of the receptor, with a time course of about 15 min. Simply detecting the endpoint as internalized or not, may not be sufficient for defining the potency of a compound as a GPCR agonist or antagonist. However, 3 time points at 5 min intervals would provide information not only about potency during the time course of measurement, but would also allow extrapolation of the data to much longer time periods. To perform this assay, the sub-region would be defined as two rows, the sampling interval as 5 minutes and the total number of time points 3. The system would then start by scanning two rows, and then adding reagent to the two rows, establishing the time=0 reference. After reagent addition, the system would again scan the two row sub-region acquiring the first time point data. Since this process would take about 250 seconds, including scanning back to the beginning of the sub-region, the system would wait 50 seconds to begin acquisition of the second time point. Two more cycles would produce the three time points and the system would move on to the second 2 row sub-region. The final two 2-row sub-regions would be scanned to finish all the wells on the plate, resulting in four time points for each well over the whole plate. Although the time points for the wells would be offset slightly relative to time=0, the spacing of the time points would be very close to the required 5 minutes, and the actual acquisition times and results recorded with much greater precision than in a fixed-cell screen.

*Example 5 High-content screen of human glucocorticoid receptor translocation*

One class of HCS involves the drug-induced dynamic redistribution of intracellular constituents. The human glucocorticoid receptor (hGR), a single “sensor” in the complex environmental response machinery of the cell, binds steroid molecules that have diffused into the cell. The ligand-receptor complex translocates to the nucleus where transcriptional activation occurs (Htun et al., *Proc. Natl. Acad. Sci.* 93:4845, 1996).

In general, hormone receptors are excellent drug targets because their activity lies at the apex of key intracellular signaling pathways. Therefore, a high-content screen of hGR translocation has distinct advantage over *in vitro* ligand-receptor binding assays. The availability of up to two more channels of fluorescence in the cell screening system of the present invention permits the screen to contain two additional parameters in parallel, such as other receptors, other distinct targets or other cellular processes.

**Plasmid construct.** A eukaryotic expression plasmid containing a coding sequence for a green fluorescent protein – human glucocorticoid receptor (GFP-hGR) chimera was prepared using GFP mutants (Palm et al., *Nat. Struct. Biol.* 4:361 (1997)). The construct was used to transfect a human cervical carcinoma cell line (HeLa).

**Cell preparation and transfection.** HeLa cells (ATCC CCL-2) were trypsinized and plated using DMEM containing 5% charcoal/dextran-treated fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (C-DMEM) 12-24 hours prior to transfection and incubated at 37°C and 5% CO<sub>2</sub>. Transfections were performed by calcium phosphate co-precipitation (Graham and Van der Eb, *Virology* 52:456, 1973; Sambrook et al., (1989). *Molecular Cloning: A Laboratory Manual*, Second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) or with Lipofectamine (Life Technologies, Gaithersburg, MD). For the calcium phosphate transfections, the medium was replaced, prior to transfection, with DMEM containing 5% charcoal/dextran-treated FBS. Cells were incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO<sub>2</sub>, washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM.

Lipofectamine transfections were performed in serum-free DMEM without antibiotics according to the manufacturer's instructions (Life Technologies,

Gaithersburg, MD). Following a 2-3 hour incubation with the DNA-liposome complexes, the medium was removed and replaced with C-DMEM. All transfected cells in 96-well microtiter plates were incubated at 33°C and 5% CO<sub>2</sub> for 24-48 hours prior to drug treatment. Experiments were performed with the receptor expressed transiently in HeLa cells.

**Dexamethasone induction of GFP-hGR translocation.** To obtain receptor-ligand translocation kinetic data, nuclei of transfected cells were first labeled with 5 µg/ml Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 33°C and 5% CO<sub>2</sub>. Cells were washed once in Hank's Balanced Salt Solution (HBSS) followed by the addition of 100 nM dexamethasone in HBSS with 1% charcoal/dextran-treated FBS. To obtain fixed time point dexamethasone titration data, transfected HeLa cells were first washed with DMEM and then incubated at 33°C and 5% CO<sub>2</sub> for 1 h in the presence of 0 – 1000 nM dexamethasone in DMEM containing 1% charcoal/dextran-treated FBS. Cells were analyzed live or they were rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis. The intracellular GFP-hGR fluorescence signal was not diminished by this fixation procedure.

**Image acquisition and analysis.** Kinetic data were collected by acquiring fluorescence image pairs (GFP-hGR and Hoechst 33342-labeled nuclei) from fields of living cells at 1 min intervals for 30 min after the addition of dexamethasone. Likewise, image pairs were obtained from each well of the fixed time point screening plates 1 h after the addition of dexamethasone. In both cases, the image pairs obtained at each time point were used to define nuclear and cytoplasmic regions in each cell. Translocation of GFP-hGR was calculated by dividing the integrated fluorescence intensity of GFP-hGR in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. In the fixed time point screen this translocation ratio was calculated from data obtained from at least 200 cells at each concentration of dexamethasone tested. Drug-induced translocation of GFP-hGR from the cytoplasm to the nucleus was therefore correlated with an increase in the translocation ratio.

**Results.** Figure 20 schematically displays the drug-induced cytoplasm to nucleus translocation of the human glucocorticoid receptor. The upper pair of

schematic diagrams depicts the localization of GFP-hGR within the cell before 250 (A) and after 251 (B) stimulation with dexamethasone. Under these experimental conditions, the drug induces a large portion of the cytoplasmic GFP-hGR to translocate into the nucleus. This redistribution is quantified by determining the integrated intensities ratio of the cytoplasmic and nuclear fluorescence in treated 255 and untreated 254 cells. The lower pair of fluorescence micrographs show the dynamic redistribution of GFP-hGR in a single cell, before 254 and after 255 treatment. The HCS is performed on wells containing hundreds to thousands of transfected cells and the translocation is quantified for each cell in the field exhibiting GFP fluorescence. Although the use of a stably transfected cell line would yield the most consistently labeled cells, the heterogeneous levels of GFP-hGR expression induced by transient transfection did not interfere with analysis by the cell screening system of the present invention.

To execute the screen, the cell screening system scans each well of the plate, images a population of cells in each, and analyzes cells individually. Here, two channels of fluorescence are used to define the cytoplasmic and nuclear distribution of the GFP-hGR within each cell. Depicted in Figure 21 is the graphical user interface of the cell screening system near the end of a GFP-hGR screen. The user interface depicts the parallel data collection and analysis capability of the system. The windows labeled "Nucleus" 261 and "GFP-hGR" 262 show the pair of fluorescence images being obtained and analyzed in a single field. The window labeled "Color Overlay" 260 is formed by pseudocoloring the above images and merging them so the user can immediately identify cellular changes. Within the "Stored Object Regions" window 265, an image containing each analyzed cell and its neighbors is presented as it is archived. Furthermore, as the HCS data are being collected, they are analyzed, in this case for GFP-hGR translocation, and translated into an immediate "hit" response. The 96 well plate depicted in the lower window of the screen 267 shows which wells have met a set of user-defined screening criteria. For example, a white-colored well 269 indicates that the drug-induced translocation has exceeded a predetermined threshold value of 50%. On the other hand, a black-colored well 270 indicates that the drug being tested induced less than 10% translocation. Gray-colored wells 268 indicate "hits" where the translocation value fell between 10% and 50%. Row "E" on the 96 well

plate being analyzed 266 shows a titration with a drug known to activate GFP-hGR translocation, dexamethasone. This example screen used only two fluorescence channels. Two additional channels (Channels 3 263 and 4 264) are available for parallel analysis of other specific targets, cell processes, or cytotoxicity to create  
5 multiple parameter screens.

There is a link between the image database and the information database that is a powerful tool during the validation process of new screens. At the completion of a screen, the user has total access to image and calculated data (Figure 22). The comprehensive data analysis package of the cell screening system allows the user to  
10 examine HCS data at multiple levels. Images 276 and detailed data in a spread sheet 279 for individual cells can be viewed separately, or summary data can be plotted. For example, the calculated results of a single parameter for each cell in a 96 well plate are shown in the panel labeled Graph 1 275. By selecting a single point in the graph, the user can display the entire data set for a particular cell that is recalled from an existing  
15 database. Shown here are the image pair 276 and detailed fluorescence and morphometric data from a single cell (Cell #118, gray line 277). The large graphical insert 278 shows the results of dexamethasone concentration on the translocation of GFP-hGR. Each point is the average of data from at least 200 cells. The calculated  $EC_{50}$  for dexamethasone in this assay is 2 nM.

A powerful aspect of HCS with the cell screening system is the capability of  
20 kinetic measurements using multicolor fluorescence and morphometric parameters in living cells. Temporal and spatial measurements can be made on single cells within a population of cells in a field. Figure 23 shows kinetic data for the dexamethasone-induced translocation of GFP-hGR in several cells within a single field. Human HeLa  
25 cells transfected with GFP-hGR were treated with 100 nM dexamethasone and the translocation of GFP-hGR was measured over time in a population of single cells. The graph shows the response of transfected cells 285, 286, 287, and 288 and non-transfected cells 289. These data also illustrate the ability to analyze cells with different expression levels.

30

*Example 6 High-content screen of drug-induced apoptosis*

Apoptosis is a complex cellular program that involves myriad molecular events and pathways. To understand the mechanisms of drug action on this process, it is essential to measure as many of these events within cells as possible with temporal and spatial resolution. Therefore, an apoptosis screen that requires little cell sample preparation yet provides an automated readout of several apoptosis-related parameters would be ideal. A cell-based assay designed for the cell screening system has been used to simultaneously quantify several of the morphological, organellar, and macromolecular hallmarks of paclitaxel-induced apoptosis.

**Cell preparation.** The cells chosen for this study were mouse connective tissue fibroblasts (L-929; ATCC CCL-1) and a highly invasive glioblastoma cell line (SNB-19; ATCC CRL-2219) (Welch et al., *In Vitro Cell. Dev. Biol.* 31:610, 1995). The day before treatment with an apoptosis inducing drug, 3500 cells were placed into each well of a 96-well plate and incubated overnight at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The following day, the culture medium was removed from each well and replaced with fresh medium containing various concentrations of paclitaxel (0 – 50 µM) from a 20 mM stock made in DMSO. The maximal concentration of DMSO used in these experiments was 0.25%. The cells were then incubated for 26 h as above. At the end of the paclitaxel treatment period, each well received fresh medium containing 750 nM MitoTracker Red (Molecular Probes; Eugene, OR) and 3 µg/ml Hoechst 33342 DNA-binding dye (Molecular Probes) and was incubated as above for 20 min. Each well on the plate was then washed with HBSS and fixed with 3.7% formaldehyde in HBSS for 15 min at room temperature. The formaldehyde was washed out with HBSS and the cells were permeabilized for 90 s with 0.5% (v/v) Triton X-100, washed with HBSS, incubated with 2 U ml<sup>-1</sup> Bodipy FL phalloidin (Molecular Probes) for 30 min, and washed with HBSS. The wells on the plate were then filled with 200 µl HBSS, sealed, and the plate stored at 4°C if necessary. The fluorescence signals from plates stored this way were stable for at least two weeks after preparation. As in the nuclear translocation assay, fluorescence reagents can be designed to convert this assay into a live cell high-content screen.

**Image acquisition and analysis on the ArrayScan System.** The fluorescence intensity of intracellular MitoTracker Red, Hoechst 33342, and Bodipy FL phalloidin

was measured with the cell screening system as described *supra*. Morphometric data from each pair of images obtained from each well was also obtained to detect each object in the image field (e.g., cells and nuclei), and to calculate its size, shape, and integrated intensity.

5           **Calculations and output.** A total of 50-250 cells were measured per image field. For each field of cells, the following calculations were performed: (1) The average nuclear area ( $\mu\text{m}^2$ ) was calculated by dividing the total nuclear area in a field by the number of nuclei detected. (2) The average nuclear perimeter ( $\mu\text{m}$ ) was calculated by dividing the sum of the perimeters of all nuclei in a field by the number  
10 of nuclei detected in that field. Highly convoluted apoptotic nuclei had the largest nuclear perimeter values. (3) The average nuclear brightness was calculated by dividing the integrated intensity of the entire field of nuclei by the number of nuclei in that field. An increase in nuclear brightness was correlated with increased DNA content. (4) The average cellular brightness was calculated by dividing the integrated intensity of an  
15 entire field of cells stained with MitoTracker dye by the number of nuclei in that field. Because the amount of MitoTracker dye that accumulates within the mitochondria is proportional to the mitochondrial potential, an increase in the average cell brightness is consistent with an increase in mitochondrial potential. (5) The average cellular brightness was also calculated by dividing the integrated intensity of an entire field of  
20 cells stained with Bodipy FL phalloidin dye by the number of nuclei in that field. Because the phalloxins bind with high affinity to the polymerized form of actin, the amount of Bodipy FL phalloidin dye that accumulates within the cell is proportional to actin polymerization state. An increase in the average cell brightness is consistent with an increase in actin polymerization.

25           **Results.** Figure 24 (top panels) shows the changes paclitaxel induced in the nuclear morphology of L-929 cells. Increasing amounts of paclitaxel caused nuclei to enlarge and fragment 293, a hallmark of apoptosis. Quantitative analysis of these and other images obtained by the cell screening system is presented in the same figure. Each parameter measured showed that the L-929 cells 296 were less sensitive to low  
30 concentrations of paclitaxel than were SNB-19 cells 297. At higher concentrations though, the L-929 cells showed a response for each parameter measured. The multiparameter approach of this assay is useful in dissecting the mechanisms of drug



action. For example, the area, brightness, and fragmentation of the nucleus 298 and actin polymerization values 294 reached a maximum value when SNB-19 cells were treated with 10 nM paclitaxel (Figure 24; top and bottom graphs). However, mitochondrial potential 295 was minimal at the same concentration of paclitaxel (Figure 24; middle graph). The fact that all the parameters measured approached control levels at increasing paclitaxel concentrations (>10 nM) suggests that SNB-19 cells have low affinity drug metabolic or clearance pathways that are compensatory at sufficiently high levels of the drug. Contrasting the drug sensitivity of SNB-19 cells 297, L-929 showed a different response to paclitaxel 296. These fibroblastic cells showed a maximal response in many parameters at 5  $\mu$ M paclitaxel, a 500-fold higher dose than SNB-19 cells. Furthermore, the L-929 cells did not show a sharp decrease in mitochondrial potential 295 at any of the paclitaxel concentrations tested. This result is consistent with the presence of unique apoptosis pathways between a normal and cancer cell line. Therefore, these results indicate that a relatively simple fluorescence labeling protocol can be coupled with the cell screening system of the present invention to produce a high-content screen of key events involved in programmed cell death.

## Background

A key to the mechanism of apoptosis was the discovery that, irrespective of the lethal stimulus, death results in identical apoptotic morphology that includes cell and organelle dismantling and repackaging, DNA cleavage to nucleosome sized fragments, and engulfment of the fragmented cell to avoid an inflammatory response. Apoptosis is therefore distinct from necrosis, which is mediated more by acute trauma to a cell, resulting in spillage of potentially toxic and antigenic cellular components into the intercellular milieu, leading to an inflammatory response.

The criteria for determining whether a cell is undergoing apoptosis (Wyllie et al. 1980. *Int Rev Cytol.* 68:251-306; Thompson, 1995. *Science.* 267:1456-62; Majno and Joris. 1995. *Am J Pathol.* 146:3-15; Allen et al. 1998. *Cell Mol Life Sci.* 54:427-45) include distinct morphological changes in the appearance of the cell, as well as alterations in biochemical and molecular markers. For example, apoptotic cells often undergo cytoplasmic membrane blebbing, their chromosomes rapidly condense and

aggregate around the nuclear periphery, the nucleus fragments, and small apoptotic bodies are formed. In many, but not all, apoptotic cells, chromatin becomes a target for specific nucleases that cleave the DNA.

Apoptosis is commonly accompanied by a characteristic change in nuclear morphology (chromatin condensation or fragmentation) and a step-wise fragmentation of DNA culminating in the formation of mono- and/or oligomeric fragments of 200 base pairs. Specific changes in organellar function, such as mitochondrial membrane potential, occur. In addition, specific cysteine proteases (caspases) are activated, which catalyzes a highly selective pattern of protein degradation by proteolytic cleavage after specific aspartic acid residues. In addition, the external surface exposure of phosphatidylserine residues (normally on the inner membrane leaflet) allows for the recognition and elimination of apoptotic cells, before the membrane breaks up and cytosol or organelles spill into the intercellular space and elicit inflammatory reactions. Moreover, cells undergoing apoptosis tend to shrink, while also having a reduced intracellular potassium level.

The general patterns of apoptotic signals are very similar among different cell types and apoptotic inducers. However, the details of the pathways actually vary significantly depending on cell type and inducer. The dependence and independence of various signal transduction pathways involved in apoptosis are currently topics of intense research. We show here that the pathway also varies depending upon the dose of the inducer in specific cell types.

### **Nuclear Morphology**

Cells undergoing apoptosis generally exhibit two types of nuclear change, fragmentation or condensation ((Majno and Joris, 1995), (Earnshaw, 1995)). The response in a given cell type appears to vary depending on the apoptotic inducer. During nuclear fragmentation, a circular or oval nucleus becomes increasingly lobular. Eventually, the nucleus fragments dramatically into multiple sub-nuclei. Sometimes the density of the chromatin within the lobular nucleus may show spatial variations in distribution (heterochromatization), approximating the margination seen in nuclear condensation.

Nuclear condensation has been reported in some cell types, such as MCF-7 (Saunders et al. 1997. *Int J Cancer*. 70:214-20). Condensation appears to arise as a consequence of the loss of structural integrity of the euchromatin, nuclear matrix and nuclear lamina (Hendzel et al. 1998. *J Biol Chem*. 273:24470-8). During nuclear  
5 condensation, the chromatin concentrates near the margin of the nucleus, leading to the overall shrinkage of the nucleus. Thus, the use of nuclear morphology as a measure of apoptosis must take both condensation and fragmentation into account.

### Material and Methods

10 Cells were plated into 96-well plates at densities of  $3 \times 10^3$  to  $1 \times 10^4$  cells/well. The following day apoptotic inducers were added at indicated concentrations and cells were incubated for indicated time periods (usually 16-30 hours). The next day medium was removed and cells were stained with 5  $\mu$ g/ml Hoechst (Molecular Probes, Inc.) in fresh medium and incubated for 30 minutes at 37°C. Cells were washed in Hank's  
15 Balanced Salt Solution (HBSS) and fixed with 3.7% formaldehyde in HBSS at room temperature. Cells were washed 2X with HBSS at room temperature and the plate was sealed.

Quantitation of changes in nuclear morphology upon induction of apoptosis was accomplished by (1) measuring the effective size of the nuclear region; and (2)  
20 measuring the degree of convolution of the perimeter. The size parameter provides the more sensitive measure of nuclear condensation, whereas the perimeter measure provides a more sensitive measure of nuclear fragmentation.

### Results & Discussion

25 L929 cells responded to both staurosporine (30 hours) and paclitaxel (30 hours) with a dose-dependent change in nuclear morphology (Fig 25A and 25B). BHK cells illustrated a slightly more complicated, yet clearly visible response. Staurosporine appeared to stimulate nuclear condensation at lower doses and nuclear fragmentation at higher doses (Fig 25C and 25D). In contrast, paclitaxel induced a consistent increase in  
30 nuclear fragmentation with increasing concentrations. The response of MCF-7 cells varied dramatically depending upon the apoptotic inducer. Staurosporine appeared to

elicit nuclear condensation whereas paclitaxel induced nuclear fragmentation (Fig 25E and 25F).

Figure 26 illustrates the dose response of cells in terms of both nuclear size and nuclear perimeter convolution. There appears to be a swelling of the nuclei that precedes the fragmentation.

**Result of evaluation:** Differential responses by cell lines and by apoptotic inducers were observed in a dose dependent manner, indicating that this assay will be useful for detecting changes in the nucleus characteristic of apoptosis.

## 10 Actin reorganization

We assessed changes in the actin cytoskeleton as a potential parameter related to apoptotic changes. This was based on preliminary observations of an early increase in f-actin content detected with fluorescent phalloidin labeling, an f-actin specific stain (our unpublished data; Levee et al. 1996. *Am J Physiol.* 271:C1981-92; Maekawa et al. 1996. *Clin Exp Immunol.* 105:389-96). Changes in the actin cytoskeleton during apoptosis have not been observed in all cell types. (Endresen et al. 1995. *Cytometry.* 20:162-71, van Engeland et al. 1997. *Exp Cell Res.* 235:421-30).

## Material and Methods

Cells were plated in 96-well plates at densities of  $3 \times 10^3$  to  $1 \times 10^4$  cells/well. The following day apoptotic inducers were added at indicated concentrations. Cells were incubated for the indicated time periods (usually 16-30 hours). The next day the medium was removed and cells were stained with 5  $\mu$ g/ml Hoechst (Molecular Probes, Inc.) in fresh medium and incubated for 30 minutes at 30°C. Cells were washed in HBSS and fixed with 3.7% formaldehyde in HBSS at room temperature. Plates were washed with HBSS and permeabilized with 0.5% v/v Triton X-100 in HBSS at room temperature. Plates were washed in HBSS and stained with 100  $\mu$ l of 1U/ml of Alexa 488 Phalloidin stock (100  $\mu$ l/well, Molecular Probes, Inc.). Cells were washed 2X with HBSS at RT and the plate was sealed.

Quantitation of f-actin content was accomplished by measuring the intensity of phalloidin staining around the nucleus. This was determined to be a reasonable approximation of a full cytoplasmic average of the intensity. The mask used to approximate this cytoplasmic measure was derived from the nuclear mask defined by

the Hoechst stain. Derivation was accomplished by combinations of erosions and dilations.

## Results and Discussion

5           Changes in f-actin content varied based on cell type and apoptotic inducer (Fig 27). Staurosporine (30 hours) induced increases in f-actin in L929 (Fig. 27A) and BHK (Fig. 27B) cells. MCF-7 cells exhibited a concentration-dependent response. At low concentrations (Fig. 27E) there appeared to be a decrease in f-actin content. At higher concentrations, f-actin content increased. Paclitaxel (30 hours) treatment led to a wide  
10       variety of responses. L929 cells responded with graded increases in f-actin (Fig. 27B) whereas both BHK and MCF-7 responses were highly variable (Figs. 27D & 27F, respectively).

**Result of Evaluation:** Both increases and decreases in signal intensity were  
15       measured for several cell lines and found to exhibit a concentration dependent response. For certain cell line/apoptotic inducer pairs this could be a statistically significant apoptotic indicator.

## Changes in Mitochondrial Mass/Potential

### 20           Introduction

          Changes in mitochondria play a central role in apoptosis (Henkart and Grinstein. 1996. *J Exp Med.* 183:1293-5). Mitochondria release apoptogenic factors through the outer membrane and dissipate the electrochemical gradient of the inner membrane. This is thought to occur via formation of the mitochondria permeability  
25       transition (MPT), although it is apparently not true in all cases. An obvious manifestation of the formation of the MPT is collapse of the mitochondrial membrane potential. Inhibition of MPT by pharmacological intervention or mitochondrial expression of the anti-apoptotic protein Bcl-2 prevents cell death, suggesting the formation of the MPT may be a rate-limiting event of the death process (For review  
30       see: Kroemer et al. 1998. *Annu Rev Physiol.* 60:619-42). It has also been observed that mitochondria can proliferate during stimulation of apoptosis (Mancini et al. 1997. *J Cell Biol.* 138:449-69; Camilleri-Broet et al. 1998. *Exp Cell Res.* 239:277-92).

One approach for measuring apoptosis-induced changes in mitochondria is to measure the mitochondrial membrane potential. Of the methods available, the simplest measure is the redistribution of a cationic dye that distributes within intracellular organelles based on the membrane potential. Such an approach traditionally requires live cells for the measurements. The recent introduction of the MitoTracker dyes (Poot et al. 1997. *Cytometry*. 27:358-64; available from Molecular Probes, Inc., Oregon) provides a means of measuring mitochondrial membrane potential after fixation.

Given the observations of a possible increase in mitochondrial mass during apoptosis, the amount of dye labeling the mitochondria is related to both membrane potential and the number of mitochondria. If the number of mitochondria remains constant then the amount of dye is directly related to the membrane potential. If the number of mitochondria is not constant, then the signal will likely be dominated by the increase in mass (Reipert et al. 1995. *Exp Cell Res*. 221:281-8).

Probes are available that allow a clear separation between changes in mass and potential in HCS assays. Mitochondrial mass is measured directly by labeling with Mitotracker Green FM (Poot and Pierce, 1999, *Cytometry*. 35:311-7; available from Molecular Probes, Inc., Oregon). The labeling is independent of mitochondrial membrane potential but proportional to mitochondrial mass. This also provides a means of normalizing other mitochondrial measures in each cell with respect to mitochondrial mass.

### Material and Methods

Cells were plated into 96-well plates at densities of  $3 \times 10^3$  to  $1 \times 10^4$  cells/well. The following day apoptotic inducers were added at the indicated concentrations and cells were incubated for the indicated time periods (usually 16-30 hours). Cells were stained with 5  $\mu\text{g/ml}$  Hoechst (Molecular Probes, Inc.) and 750 nM MitoTracker Red (CMXRos, Molecular Probes, Inc.) in fresh medium and incubated for 30 minutes at 37°C. Cells were washed in HBSS and fixed with 3.7% formaldehyde in HBSS at room temperature. Plates were washed with HBSS and permeabilized with 0.5% v/v Triton X-100 in HBSS at room temperature. Cells were washed 2X with HBSS at room temperature and the plate was sealed. For dual labeling of mitochondria, cells were

treated with 200 nM Mitotracker Green and 200 nM Mitotracker Red for 0.5 hours before fixation.

## Results & Discussion

5 Induction of apoptosis by staurosporine and paclitaxel led to varying mitochondrial changes depending upon the stimulus. L929 cells exhibited a clear increase in mitochondrial mass with increasing staurosporine concentrations (Fig. 28). BHK cells exhibited either a decrease in membrane potential at lower concentrations of staurosporine, or an increase in mass at higher concentrations of staurosporine (Fig. 10 28C). MCF-7 cells responded by a consistent decrease in mitochondrial membrane potential in response to increasing concentrations of staurosporine (Fig 28E). Increasing concentrations of paclitaxel caused consistent increases in mitochondrial mass (Fig 28B, 28D, and 28F).

The mitochondrial membrane potential is measured by labeling mitochondria 15 with both Mitotracker Green FM and Mitotracker Red (Molecular Probes, Inc). Mitotracker Red labeling is proportional to both mass and membrane potential. Mitotracker Green FM labeling is proportional to mass. The ratio of Mitotracker Red signal to the Mitotracker Green FM signal provides a measure of mitochondrial membrane potential (Poot and Pierce, 1999). This ratio normalizes the mitochondrial 20 mass with respect to the Mitotracker Red signal. (See Figure 28G) Combining the ability to normalize to mitochondrial mass with a measure of the membrane potential allows independent assessment of both parameters.

**Result of Evaluation:** Both decreases in potential and increases in mass were observed 25 depending on the cell line and inducer tested. Dose dependent correlation demonstrates that this is a promising apoptotic indicator.

It is possible to combine multiple measures of apoptosis by exploiting the spectral domain of fluorescence spectroscopy. In fact, all of the nuclear morphology/f-actin content/mitochondrial mass/mitochondrial potential data shown earlier were 30 collected as multiparameter assays, but were presented individually for clarity.

*Example 7. Protease induced translocation of a signaling enzyme containing a disease-associated sequence from cytoplasm to nucleus.*

**Plasmid construct.** A eukaryotic expression plasmid containing a coding  
5 sequence for a green fluorescent protein – caspase (Cohen (1997), *Biochemical J.*  
326:1-16; Liang et al. (1997), *J. of Molec. Biol.* 274:291-302) chimera is prepared using  
GFP mutants. The construct is used to transfect eukaryotic cells.

**Cell preparation and transfection.** Cells are trypsinized and plated 24 h prior  
to transfection and incubated at 37°C and 5% CO<sub>2</sub>. Transfections are performed by  
10 methods including, but not limited to calcium phosphate coprecipitation or lipofection.  
Cells are incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C  
and 5% CO<sub>2</sub>, washed 3-4 times with DMEM to remove the precipitate, followed by the  
addition of C-DMEM. Lipofectamine transfections are performed in serum-free  
DMEM without antibiotics according to the manufacturer's instructions. Following a  
15 2-3 hour incubation with the DNA-liposome complexes, the medium is removed and  
replaced with C-DMEM.

**Apoptotic induction of Caspase-GFP translocation.** To obtain Caspase-GFP  
translocation kinetic data, nuclei of transfected cells are first labeled with 5 µg/ml  
Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 37°C and 5% CO<sub>2</sub>.  
20 Cells are washed once in Hank's Balanced Salt Solution (HBSS) followed by the  
addition of compounds that induce apoptosis. These compounds include, but are not  
limited to paclitaxel, staurosporine, ceramide, and tumor necrosis factor. To obtain  
fixed time point titration data, transfected cells are first washed with DMEM and then  
incubated at 37°C and 5% CO<sub>2</sub> for 1 h in the presence of 0 – 1000 nM compound in  
25 DMEM. Cells are analyzed live or they are rinsed with HBSS, fixed for 15 min with  
3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis.

**Image acquisition and analysis.** Kinetic data are collected by acquiring  
fluorescence image pairs (Caspase-GFP and Hoechst 33342-labeled nuclei) from fields  
of living cells at 1 min intervals for 30 min after the addition of compound. Likewise,  
30 image pairs are obtained from each well of the fixed time point screening plates 1 h  
after the addition of compound. In both cases, the image pairs obtained at each time  
point are used to define nuclear and cytoplasmic regions in each cell. Translocation of



Caspase-GFP is calculated by dividing the integrated fluorescence intensity of Caspase-GFP in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. In the fixed time point screen this translocation ratio is calculated from data obtained from at least 200 cells at each concentration of compound tested. Drug-induced translocation of Caspase-GFP from the cytoplasm to the nucleus is therefore correlated with an increase in the translocation ratio. Molecular interaction libraries including, but not limited to those comprising putative activators or inhibitors of apoptosis-activated enzymes are used to screen the indicator cell lines and identify a specific ligand for the DAS, and a pathway activated by compound activity.

*Example 8. Identification of novel steroid receptors from DAS*

Two sources of material and/or information are required to make use of this embodiment, which allows assessment of the function of an uncharacterized gene. First, disease associated sequence bank(s) containing cDNA sequences suitable for transfection into mammalian cells can be used. Because every RADE or differential expression experiment generates up to several hundred sequences, it is possible to generate an ample supply of DAS. Second, information from primary sequence database searches can be used to place DAS into broad categories, including, but not limited to, those that contain signal sequences, seven trans-membrane motifs, conserved protease active site domains, or other identifiable motifs. Based on the information acquired from these sources, method types and indicator cell lines to be transfected are selected. A large number of motifs are already well characterized and encoded in the linear sequences contained within the large number genes in existing genomic databases.

In one embodiment, the following steps are taken:

1) Information from the DAS identification experiment (including database searches) is used as the basis for selecting the relevant biological processes. (for example, look at the DAS from a tumor line for cell cycle modulation, apoptosis, metastatic proteases, etc.)

2) Sorting of DNA sequences or DAS by identifiable motifs (ie. signal sequences, 7- transmembrane domains, conserved protease active site domains, etc.) This initial grouping will determine fluorescent tagging strategies, host cell lines,

indicator cell lines, and banks of bioactive molecules to be screened, as described *supra*.

5           3) Using well established molecular biology methods, ligate DAS into an expression vector designed for this purpose. Generalized expression vectors contain promoters, enhancers, and terminators for which to deliver target sequences to the cell for transient expression. Such vectors may also contain antibody tagging sequences, direct association sequences, chromophore fusion sequences like GFP, etc. to facilitate detection when expressed by the host.

10           4) Transiently transfect cells with DAS containing vectors using standard transfection protocols including: calcium phosphate co-precipitation, liposome mediated, DEAE dextran mediated, polycationic mediated, viral mediated, or electroporation, and plate into microtiter plates or microwell arrays. Alternatively, transfection can be done directly in the microtiter plate itself.

15           5) Carry out the cell screening methods as described *supra*.

In this embodiment, DAS shown to possess a motif(s) suggestive of transcriptional activation potential (for example, DNA binding domain, amino terminal modulating domain, hinge region, or carboxy terminal ligand binding domain) are utilized to identify novel steroid receptors.

20           Defining the fluorescent tags for this experiment involves identification of the nucleus through staining, and tagging the DAS by creating a GFP chimera via insertion of DAS into an expression vector, proximally fused to the gene encoding GFP. Alternatively, a single chain antibody fragment with high affinity to some portion of the expressed DAS could be constructed using technology available in the art (Cambridge  
25   Antibody Technologies) and linked to a fluorophore (FITC) to tag the putative transcriptional activator/receptor in the cells. This alternative would provide an external tag requiring no DNA transfection and therefore would be useful if distribution data were to be gathered from the original primary cultures used to generate the DAS.

**Plasmid construct.** A eukaryotic expression plasmid containing a coding  
30   sequence for a green fluorescent protein – DAS chimera is prepared using GFP mutants. The construct is used to transfect HeLa cells. The plasmid, when transfected into the host cell, produces a GFP fused to the DAS protein product, designated GFP-DASpp.

**Cell preparation and transfection.** HeLa cells are trypsinized and plated using DMEM containing 5% charcoal/dextran-treated fetal bovine serum (FBS) (Hyclone) and 1% penicillin-streptomycin (C-DMEM) 12-24 hours prior to transfection and incubated at 37°C and 5% CO<sub>2</sub>. Transfections are performed by calcium phosphate coprecipitation or with Lipofectamine (Life Technologies). For the calcium phosphate transfections, the medium is replaced, prior to transfection, with DMEM containing 5% charcoal/dextran-treated FBS. Cells are incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO<sub>2</sub>, and washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM. Lipofectamine transfections are performed in serum-free DMEM without antibiotics according to the manufacturer's instructions. Following a 2-3 hour incubation with the DNA-liposome complexes, the medium is removed and replaced with C-DMEM. All transfected cells in 96-well microtiter plates are incubated at 33°C and 5% CO<sub>2</sub> for 24-48 hours prior to drug treatment. Experiments are performed with the receptor expressed transiently in HeLa cells.

**Localization of expressed GFP-DASpp inside cells.** To obtain cellular distribution data, nuclei of transfected cells are first labeled with 5 µg/ml Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 33°C and 5% CO<sub>2</sub>. Cells are washed once in Hank's Balanced Salt Solution (HBSS). The cells are analyzed live or they are rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis.

In a preferred embodiment, image acquisition and analysis are performed using the cell screening system of the present invention. The intracellular GFP-DASpp fluorescence signal is collected by acquiring fluorescence image pairs (GFP-DASpp and Hoechst 33342-labeled nuclei) from field cells. The image pairs obtained at each time point are used to define nuclear and cytoplasmic regions in each cell. Data demonstrating dispersed signal in the cytoplasm would be consistent with known steroid receptors that are DNA transcriptional activators.

**Screening for induction of GFP-DASpp translocation.** Using the above construct, confirmed for appropriate expression of the GFP-DASpp, as an indicator cell line, a screen of various ligands is performed using a series of steroid type ligands including, but not limited to: estrogen, progesterone, retinoids, growth factors,

androgens, and many other steroid and steroid based molecules. Image acquisition and analysis are performed using the cell screening system of the invention. The intracellular GFP-DASpp fluorescence signal is collected by acquiring fluorescence image pairs (GFP-DASpp and Hoechst 33342-labeled nuclei) from fields cells. The image pairs obtained at each time point are used to define nuclear and cytoplasmic regions in each cell. Translocation of GFP-DASpp is calculated by dividing the integrated fluorescence intensity of GFP-DASpp in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. A translocation from the cytoplasm into the nucleus indicates a ligand binding activation of the DASpp thus identifying the potential receptor class and action. Combining this data with other data obtained in a similar fashion using known inhibitors and modifiers of steroid receptors, would either validate the DASpp as a target, or more data would be generated from various sources.

#### Example 9 Additional Screens

##### *Translocation between the plasma membrane and the cytoplasm:*

**Profilactin complex dissociation and binding of profilin to the plasma membrane.** In one embodiment, a fluorescent protein biosensor of profilin membrane binding is prepared by labeling purified profilin (Federov et al.(1994), *J. Molec. Biol.* 241:480-482; Lanbrechts et al. (1995), *Eur. J. Biochem.* 230:281-286) with a probe possessing a fluorescence lifetime in the range of 2-300 ns. The labeled profilin is introduced into living indicator cells using bulk loading methodology and the indicator cells are treated with test compounds. Fluorescence anisotropy imaging microscopy (Gough and Taylor (1993), *J. Cell Biol.* 121:1095-1107) is used to measure test-compound dependent movement of the fluorescent derivative of profilin between the cytoplasm and membrane for a period of time after treatment ranging from 0.1 s to 10 h.

**Rho-RhoGDI complex translocation to the membrane.** In another embodiment, indicator cells are treated with test compounds and then fixed, washed, and permeabilized. The indicator cell plasma membrane, cytoplasm, and nucleus are all labeled with distinctly colored markers followed by immunolocalization of Rho protein (Self et al. (1995), *Methods in Enzymology* 256:3-10; Tanaka et al. (1995),

*Methods in Enzymology* 256:41-49) with antibodies labeled with a fourth color. Each of the four labels is imaged separately using the cell screening system, and the images used to calculate the amount of inhibition or activation of translocation effected by the test compound. To do this calculation, the images of the probes used to mark the plasma membrane and cytoplasm are used to mask the image of the immunological probe marking the location of intracellular Rho protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the plasma membrane integrated brightness/area by the cytoplasmic integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound.

*$\beta$ -Arrestin translocation to the plasma membrane upon G-protein receptor activation.*

In another embodiment of a cytoplasm to membrane translocation high-content screen, the translocation of  $\beta$ -arrestin protein from the cytoplasm to the plasma membrane is measured in response to cell treatment. To measure the translocation, living indicator cells containing luminescent domain markers are treated with test compounds and the movement of the  $\beta$ -arrestin marker is measured in time and space using the cell screening system of the present invention. In a preferred embodiment, the indicator cells contain luminescent markers consisting of a green fluorescent protein  $\beta$ -arrestin (GFP- $\beta$ -arrestin) protein chimera (Barak et al. (1997), *J. Biol. Chem.* 272:27497-27500; Daaka et al. (1998), *J. Biol. Chem.* 273:685-688) that is expressed by the indicator cells through the use of transient or stable cell transfection and other reporters used to mark cytoplasmic and membrane domains. When the indicator cells are in the resting state, the domain marker molecules partition predominately in the plasma membrane or in the cytoplasm. In the high-content screen, these markers are used to delineate the cell cytoplasm and plasma membrane in distinct channels of fluorescence. When the indicator cells are treated with a test compound, the dynamic redistribution of the GFP- $\beta$ -arrestin is recorded as a series of images over a time scale ranging from 0.1 s to 10 h. In a preferred embodiment, the time scale is 1 h. Each image is analyzed by a method that quantifies the movement of the GFP- $\beta$ -arrestin

protein chimera between the plasma membrane and the cytoplasm. To do this calculation, the images of the probes used to mark the plasma membrane and cytoplasm are used to mask the image of the GFP- $\beta$ -arrestin probe marking the location of intracellular GFP- $\beta$ -arrestin protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the plasma membrane integrated brightness/area by the cytoplasmic integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compounds of interest.

*Translocation between the endoplasmic reticulum and the Golgi:*

In one embodiment of an endoplasmic reticulum to Golgi translocation high-content screen, the translocation of a VSVG protein from the ts045 mutant strain of vesicular stomatitis virus (Ellenberg et al. (1997), *J. Cell Biol.* 138:1193-1206; Presley et al. (1997) *Nature* 389:81-85) from the endoplasmic reticulum to the Golgi domain is measured in response to cell treatment. To measure the translocation, indicator cells containing luminescent reporters are treated with test compounds and the movement of the reporters is measured in space and time using the cell screening system of the present invention. The indicator cells contain luminescent reporters consisting of a GFP-VSVG protein chimera that is expressed by the indicator cell through the use of transient or stable cell transfection and other domain markers used to measure the localization of the endoplasmic reticulum and Golgi domains. When the indicator cells are in their resting state at 40°C, the GFP-VSVG protein chimera molecules are partitioned predominately in the endoplasmic reticulum. In this high-content screen, domain markers of distinct colors used to delineate the endoplasmic reticulum and the Golgi domains in distinct channels of fluorescence. When the indicator cells are treated with a test compound and the temperature is simultaneously lowered to 32°C, the dynamic redistribution of the GFP-VSVG protein chimera is recorded as a series of images over a time scale ranging from 0.1 s to 10 h. Each image is analyzed by a method that quantifies the movement of the GFP-VSVG protein chimera between the endoplasmic reticulum and the Golgi domains. To do this calculation, the images of

the probes used to mark the endoplasmic reticulum and the Golgi domains are used to mask the image of the GFP-VSVG probe marking the location of intracellular GFP-VSVG protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the endoplasmic reticulum integrated brightness/area by the Golgi integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compounds of interest at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for a period ranging from 1 min to 10 h.

*Induction and inhibition of organellar function:*

**Intracellular microtubule stability.**

In another aspect of the invention, an automated method for identifying compounds that modify microtubule structure is provided. In this embodiment, indicator cells are treated with test compounds and the distribution of luminescent microtubule-labeling molecules is measured in space and time using a cell screening system, such as the one disclosed above. The luminescent microtubule-labeling molecules may be expressed by or added to the cells either before, together with, or after contacting the cells with a test compound.

In one embodiment of this aspect of the invention, living cells express a luminescently labeled protein biosensor of microtubule dynamics, comprising a protein that labels microtubules fused to a luminescent protein. Appropriate microtubule-labeling proteins for this aspect of the invention include, but are not limited to  $\alpha$  and  $\beta$  tubulin isoforms, and MAP4. Preferred embodiments of the luminescent protein include, but are not limited to green fluorescent protein (GFP) and GFP mutants. In a preferred embodiment, the method involves transfecting cells with a microtubule labeling luminescent protein, wherein the microtubule labeling protein can be, but is not limited to,  $\alpha$ -tubulin,  $\beta$ -tubulin, or microtubule-associated protein 4 (MAP4). The approach outlined here enables those skilled in the art to make live cell measurements

to determine the effect of lead compounds on tubulin activity and microtubule stability *in vivo*.

In a most preferred embodiment, MAP4 is fused to a modified version of the *Aequorea victoria* green fluorescent protein (GFP). A DNA construct has been made which consists of a fusion between the EGFP coding sequence (available from Clontech) and the coding sequence for mouse MAP4. (Olson et al., (1995), J. Cell Biol. 130(3): 639-650). MAP4 is a ubiquitous microtubule-associated protein that is known to interact with microtubules in interphase as well as mitotic cells (Olmsted and Murofushi, (1993), MAP4. In "Guidebook to the Cytoskeleton and Motor Proteins." Oxford University Press. T. Kreis and R. Vale, eds.) Its localization, then, can serve as an indicator of the localization, organization, and integrity of microtubules in living (or fixed) cells at all stages of the cell cycle for cell-based HCS assays. While MAP2 and tau (microtubule associated proteins expressed specifically in neuronal cells) have been used to form GFP chimeras (Kaech et al., (1996) Neuron. 17: 1189-1199; Hall et al., (1997), Proc. Nat. Acad. Sci. 94: 4733-4738) their restricted cell type distribution and the tendency of these proteins to bundle microtubules when overexpressed make these proteins less desirable as molecular reagents for analysis in live cells originating from varied tissues and organs. Moderate overexpression of GFP-MAP4 does not disrupt microtubule function or integrity (Olson et al., 1995). Similar constructs can be made using  $\beta$ -tubulin or  $\alpha$ -tubulin via standard techniques in the art. These chimeras will provide a means to observe and analyze microtubule activity in living cells during all stages of the cell cycle.

In another embodiment, the luminescently labeled protein biosensor of microtubule dynamics is expressed, isolated, and added to the cells to be analyzed via bulk loading techniques, such as microinjection, scrape loading, and impact-mediated loading. In this embodiment, there is not an issue of overexpression within the cell, and thus  $\alpha$  and  $\beta$  tubulin isoforms, MAP4, MAP2 and/or tau can all be used.

In a further embodiment, the protein biosensor is expressed by the cell, and the cell is subsequently contacted with a luminescent label, such as a labeled antibody, that detects the protein biosensor, endogenous levels of a protein antigen, or both. In this embodiment, a luminescent label that detects  $\alpha$  and  $\beta$  tubulin isoforms, MAP4, MAP2 and/or tau, can be used.



A variety of GFP mutants are available, all of which would be effective in this invention, including, but not limited to, GFP mutants which are commercially available (Clontech, California).

The MAP4 construct has been introduced into several mammalian cell lines (BHK-21, Swiss 3T3, HeLa, HEK 293, LLCPK) and the organization and localization of tubulin has been visualized in live cells by virtue of the GFP fluorescence as an indicator of MAP4 localization. The construct can be expressed transiently or stable cell lines can be prepared by standard methods. Stable HeLa cell lines expressing the EGFP-MAP4 chimera have been obtained, indicating that expression of the chimera is not toxic and does not interfere with mitosis.

Possible selectable markers for establishment and maintenance of stable cell lines include, but are not limited to the neomycin resistance gene, hygromycin resistance gene, zeocin resistance gene, puromycin resistance gene, bleomycin resistance gene, and blastacidin resistance gene.

The utility of this method for the monitoring of microtubule assembly, disassembly, and rearrangement has been demonstrated by treatment of transiently and stably transfected cells with microtubule drugs such as paclitaxel, nocodazole, vincristine, or vinblastine.

The present method provides high-content and combined high throughput-high content cell-based screens for anti-microtubule drugs, particularly as one parameter in a multi-parametric cancer target screen. The EGFP-MAP4 construct used herein can also be used as one of the components of a high-content screen that measures multiple signaling pathways or physiological events. In a preferred embodiment, a combined high throughput and high content screen is employed, wherein multiple cells in each of the locations containing cells are analyzed in a high throughput mode, and only a subset of the locations containing cells are analyzed in a high content mode. The high throughput screen can be any screen that would be useful to identify those locations containing cells that should be further analyzed, including, but not limited to, identifying locations with increased luminescence intensity, those exhibiting expression of a reporter gene, those undergoing calcium changes, and those undergoing pH changes.

In addition to drug screening applications, the present invention may be applied to clinical diagnostics, the detection of chemical and biological warfare weapons, and the basic research market since fundamental cell processes, such as cell division and motility, are highly dependent upon microtubule dynamics.

5

### *Image Acquisition and Analysis*

Image data can be obtained from either fixed or living indicator cells. To extract morphometric data from each of the images obtained the following method of analysis is used:

- 10 1. Threshold each nucleus and cytoplasmic image to produce a mask that has value = 0 for each pixel outside a nucleus or cell boundary.
2. Overlay the mask on the original image, detect each object in the field (*i.e.*, nucleus or cell), and calculate its size, shape, and integrated intensity.
- 15 3. Overlay the whole cell mask obtained above on the corresponding luminescent microtubule image and apply one or more of the following set of classifiers to determine the microtubule morphology and the effect of drugs on microtubule morphology.

Microtubule morphology is defined using a set of classifiers to quantify aspects of microtubule shape, size, aggregation state, and polymerization state. These  
20 classifiers can be based on approaches that include co-occurrence matrices, texture measurements, spectral methods, structural methods, wavelet transforms, statistical methods, or combinations thereof. Examples of such classifiers are as follows:

1. A classifier to quantify microtubule length and width using edge detection methods such as that discussed in Kolega et al. ((1993). *BioImaging* 1:136-150), which discloses a non-automated method to determine edge strength in individual  
25 cells), to calculate the total edge strength within each cell. To normalize for cell size, the total edge strength can be divided by the cell area to give a "microtubule morphology" value. Large microtubule morphology values are associated with strong edge strength values and are therefore maximal in cells containing distinct microtubule structures. Likewise, small microtubule morphology values are associated with weak  
30 edge strength and are minimal in cells with depolymerized microtubules. The physiological range of microtubule morphology values is set by treating cells with either the microtubule stabilizing drug paclitaxel (10  $\mu$ M) or the microtubule depolymerizing drug nocodazole (10  $\mu$ g/ml).

35

2. A classifier to quantify microtubule aggregation into punctate spots or foci using methodology from the receptor internalization methods discussed supra.

3. A classifier to quantify microtubule depolymerization using a measure of image texture.

5 4. A classifier to quantify apparent interconnectivity, or branching (or both), of the microtubules.

5. Measurement of the kinetics of microtubule reorganization using the above classifiers on a time series of images of cells treated with test compounds.

10

In a further aspect, kits are provided for analyzing microtubule stability, comprising an expression vector comprising a nucleic acid that encodes a microtubule labeling protein and instructions for using the expression vector for carrying out the methods described above. In a preferred embodiment, the expression vector further  
15 comprises a nucleic acid that encodes a luminescent protein, wherein the microtubule binding protein and the luminescent protein thereof are expressed as a fusion protein. Alternatively, the kit may contain an antibody that specifically binds to the microtubule-labeling protein. In a further embodiment, the kit includes cells that express the microtubule labeling protein. In a preferred embodiment, the cells are  
20 transfected with the expression vector. In another preferred embodiment, the kits further contain a compound that is known to disrupt microtubule structure, including but not limited to curacin, nocodazole, vincristine, or vinblastine. In another preferred embodiment, the kits further comprise a compound that is known to stabilize microtubule structure, including but not limited to taxol (paclitaxel), and  
25 discodermolide.

In another aspect, the present invention comprises a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the disclosed methods for analyzing microtubule stability, wherein the cell screening system comprises an optical system with a stage adapted for  
30 holding a plate containing cells, a digital camera, a means for directing fluorescence or luminescence emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera.

*High-content screens involving the functional localization of macromolecules*

Within this class of high-content screen, the functional localization of macromolecules in response to external stimuli is measured within living cells.

**Glycolytic enzyme activity regulation.** In a preferred embodiment of a cellular enzyme activity high-content screen, the activity of key glycolytic regulatory enzymes are measured in treated cells. To measure enzyme activity, indicator cells containing luminescent labeling reagents are treated with test compounds and the activity of the reporters is measured in space and time using cell screening system of the present invention.

In one embodiment, the reporter of intracellular enzyme activity is fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase (PFK-2), a regulatory enzyme whose phosphorylation state indicates intracellular carbohydrate anabolism or catabolism (Deprez et al. (1997) *J. Biol. Chem.* 272:17269-17275; Kealer et al. (1996) *FEBS Letters* 395:225-227; Lee et al. (1996), *Biochemistry* 35:6010-6019). The indicator cells contain luminescent reporters consisting of a fluorescent protein biosensor of PFK-2 phosphorylation. The fluorescent protein biosensor is constructed by introducing an environmentally sensitive fluorescent dye near to the known phosphorylation site of the enzyme (Deprez et al. (1997), *supra*; Giuliano et al. (1995), *supra*). The dye can be of the ketocyanine class (Kessler and Wolfbeis (1991), *Spectrochimica Acta* 47A:187-192 ) or any class that contains a protein reactive moiety and a fluorochrome whose excitation or emission spectrum is sensitive to solution polarity. The fluorescent protein biosensor is introduced into the indicator cells using bulk loading methodology.

Living indicator cells are treated with test compounds, at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells by collecting a spectral pair of fluorescence images at each time point. To extract morphometric data from each time point, a ratio is made between each pair of images by numerically dividing the two spectral images at each time point, pixel by pixel. Each pixel value is then used to calculate the fractional phosphorylation of PFK-2. At small fractional values of phosphorylation, PFK-2 stimulates carbohydrate catabolism.

At high fractional values of phosphorylation, PFK-2 stimulates carbohydrate anabolism.

**Protein kinase A activity and localization of subunits.** In another embodiment of a high-content screen, both the domain localization and activity of protein kinase A (PKA) within indicator cells are measured in response to treatment with test compounds.

The indicator cells contain luminescent reporters including a fluorescent protein biosensor of PKA activation. The fluorescent protein biosensor is constructed by introducing an environmentally sensitive fluorescent dye into the catalytic subunit of PKA near the site known to interact with the regulatory subunit of PKA (Harootunian et al. (1993), *Mol. Biol. of the Cell* 4:993-1002; Johnson et al. (1996), *Cell* 85:149-158; Giuliano et al. (1995), *supra*). The dye can be of the ketocyanine class (Kessler, and Wolfbeis (1991), *Spectrochimica Acta* 47A:187-192) or any class that contains a protein reactive moiety and a fluorochrome whose excitation or emission spectrum is sensitive to solution polarity. The fluorescent protein biosensor of PKA activation is introduced into the indicator cells using bulk loading methodology.

In one embodiment, living indicator cells are treated with test compounds, at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells. To extract biosensor data from each time point, a ratio is made between each pair of images, and each pixel value is then used to calculate the fractional activation of PKA (e.g., separation of the catalytic and regulatory subunits after cAMP binding). At high fractional values of activity, PFK-2 stimulates biochemical cascades within the living cell.

To measure the translocation of the catalytic subunit of PKA, indicator cells containing luminescent reporters are treated with test compounds and the movement of the reporters is measured in space and time using the cell screening system. The indicator cells contain luminescent reporters consisting of domain markers used to measure the localization of the cytoplasmic and nuclear domains. When the indicator cells are treated with a test compounds, the dynamic redistribution of a PKA fluorescent protein biosensor is recorded intracellularly as a series of images over a

time scale ranging from 0.1 s to 10 h. Each image is analyzed by a method that quantifies the movement of the PKA between the cytoplasmic and nuclear domains. To do this calculation, the images of the probes used to mark the cytoplasmic and nuclear domains are used to mask the image of the PKA fluorescent protein biosensor. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the cytoplasmic integrated brightness/area by the nuclear integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compound in the concentration range of  $10^{-12}$  M to  $10^{-3}$  M.

*High-content screens involving the induction or inhibition of gene expression*

*RNA-based fluorescent biosensors*

**Cytoskeletal protein transcription and message localization.** Regulation of the general classes of cell physiological responses including cell-substrate adhesion, cell-cell adhesion, signal transduction, cell-cycle events, intermediary and signaling molecule metabolism, cell locomotion, cell-cell communication, and cell death can involve the alteration of gene expression. High-content screens can also be designed to measure this class of physiological response.

In one embodiment, the reporter of intracellular gene expression is an oligonucleotide that can hybridize with the target mRNA and alter its fluorescence signal. In a preferred embodiment, the oligonucleotide is a molecular beacon (Tyagi and Kramer (1996) *Nat. Biotechnol.* 14:303-308), a luminescence-based reagent whose fluorescence signal is dependent on intermolecular and intramolecular interactions. The fluorescent biosensor is constructed by introducing a fluorescence energy transfer pair of fluorescent dyes such that there is one at each end (5' and 3') of the reagent. The dyes can be of any class that contains a protein reactive moiety and fluorochromes whose excitation and emission spectra overlap sufficiently to provide fluorescence energy transfer between the dyes in the resting state, including, but not limited to, fluorescein and rhodamine (Molecular Probes, Inc.). In a preferred embodiment, a

portion of the message coding for  $\beta$ -actin (Kislauskis et al. (1994), *J. Cell Biol.* 127:441-451; McCann et al. (1997), *Proc. Natl. Acad. Sci.* 94:5679-5684; Sutoh (1982), *Biochemistry* 21:3654-3661) is inserted into the loop region of a hairpin-shaped oligonucleotide with the ends tethered together due to intramolecular hybridization. At each end of the biosensor a fluorescence donor (fluorescein) and a fluorescence acceptor (rhodamine) are covalently bound. In the tethered state, the fluorescence energy transfer is maximal and therefore indicative of an unhybridized molecule. When hybridized with the mRNA coding for  $\beta$ -actin, the tether is broken and energy transfer is lost. The complete fluorescent biosensor is introduced into the indicator cells using bulk loading methodology.

In one embodiment, living indicator cells are treated with test compounds, at final concentrations ranging from  $10^{-12}$  M to  $10^{-3}$  M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells. To extract morphometric data from each time point, a ratio is made between each pair of images, and each pixel value is then used to calculate the fractional hybridization of the labeled nucleotide. At small fractional values of hybridization little expression of  $\beta$ -actin is indicated. At high fractional values of hybridization, maximal expression of  $\beta$ -actin is indicated. Furthermore, the distribution of hybridized molecules within the cytoplasm of the indicator cells is also a measure of the physiological response of the indicator cells.

#### *Cell surface binding of a ligand*

**Labeled insulin binding to its cell surface receptor in living cells.** Cells whose plasma membrane domain has been labeled with a labeling reagent of a particular color are incubated with a solution containing insulin molecules (Lee et al. (1997), *Biochemistry* 36:2701-2708; Martinez-Zaguilan et al. (1996), *Am. J. Physiol.* 270:C1438-C1446) that are labeled with a luminescent probe of a different color for an appropriate time under the appropriate conditions. After incubation, unbound insulin molecules are washed away, the cells fixed and the distribution and concentration of the insulin on the plasma membrane is measured. To do this, the cell membrane image is used as a mask for the insulin image. The integrated intensity from the masked insulin image is compared to a set of images containing known amounts of labeled insulin.

The amount of insulin bound to the cell is determined from the standards and used in conjunction with the total concentration of insulin incubated with the cell to calculate a dissociation constant or insulin to its cell surface receptor.

5    *Labeling of cellular compartments*

**Whole cell labeling**

      Whole cell labeling is accomplished by labeling cellular components such that dynamics of cell shape and motility of the cell can be measured over time by analyzing fluorescence images of cells.

10        In one embodiment, small reactive fluorescent molecules are introduced into living cells. These membrane-permeant molecules both diffuse through and react with protein components in the plasma membrane. Dye molecules react with intracellular molecules to both increase the fluorescence signal emitted from each molecule and to entrap the fluorescent dye within living cells. These molecules include reactive  
15    chloromethyl derivatives of aminocoumarins, hydroxycoumarins, eosin diacetate, fluorescein diacetate, some Bodipy dye derivatives, and tetramethylrhodamine. The reactivity of these dyes toward macromolecules includes free primary amino groups and free sulfhydryl groups.

      In another embodiment, the cell surface is labeled by allowing the cell to  
20    interact with fluorescently labeled antibodies or lectins (Sigma Chemical Company, St. Louis, MO) that react specifically with molecules on the cell surface. Cell surface protein chimeras expressed by the cell of interest that contain a green fluorescent protein, or mutant thereof, component can also be used to fluorescently label the entire cell surface. Once the entire cell is labeled, images of the entire cell or cell array can  
25    become a parameter in high content screens, involving the measurement of cell shape, motility, size, and growth and division.

**Plasma membrane labeling**

30        In one embodiment, labeling the whole plasma membrane employs some of the same methodology described above for labeling the entire cells. Luminescent molecules that label the entire cell surface act to delineate the plasma membrane.



In a second embodiment subdomains of the plasma membrane, the extracellular surface, the lipid bilayer, and the intracellular surface can be labeled separately and used as components of high content screens. In the first embodiment, the extracellular surface is labeled using a brief treatment with a reactive fluorescent molecule such as the succinimidyl ester or iodoacetamide derivatives of fluorescent dyes such as the fluoresceins, rhodamines, cyanines, and Bodipys.

In a third embodiment, the extracellular surface is labeled using fluorescently labeled macromolecules with a high affinity for cell surface molecules. These include fluorescently labeled lectins such as the fluorescein, rhodamine, and cyanine derivatives of lectins derived from jack bean (Con A), red kidney bean (erythroagglutinin PHA-E), or wheat germ.

In a fourth embodiment, fluorescently labeled antibodies with a high affinity for cell surface components are used to label the extracellular region of the plasma membrane. Extracellular regions of cell surface receptors and ion channels are examples of proteins that can be labeled with antibodies.

In a fifth embodiment, the lipid bilayer of the plasma membrane is labeled with fluorescent molecules. These molecules include fluorescent dyes attached to long chain hydrophobic molecules that interact strongly with the hydrophobic region in the center of the plasma membrane lipid bilayer. Examples of these dyes include the PKH series of dyes (U.S. 4,783,401, 4,762,701, and 4,859,584; available commercially from Sigma Chemical Company, St. Louis, MO), fluorescent phospholipids such as nitrobenzoxadiazole glycerophosphoethanolamine and fluorescein-derivatized dihexadecanoylglycerophosphoethanolamine, fluorescent fatty acids such as 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoic acid and 1-pyrenedecanoic acid (Molecular Probes, Inc.), fluorescent sterols including cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate and cholesteryl 1-pyrenehexanoate, and fluorescently labeled proteins that interact specifically with lipid bilayer components such as the fluorescein derivative of annexin V (Caltag Antibody Co, Burlingame, CA).

In another embodiment, the intracellular component of the plasma membrane is labeled with fluorescent molecules. Examples of these molecules are the intracellular components of the trimeric G-protein receptor, adenylyl cyclase, and ionic transport

proteins. These molecules can be labeled as a result of tight binding to a fluorescently labeled specific antibody or by the incorporation of a fluorescent protein chimera that is comprised of a membrane-associated protein and the green fluorescent protein, and mutants thereof.

5

### Endosome fluorescence labeling

In one embodiment, ligands that are transported into cells by receptor-mediated endocytosis are used to trace the dynamics of endosomal organelles. Examples of labeled ligands include Bodipy FL-labeled low density lipoprotein complexes, tetramethylrhodamine transferrin analogs, and fluorescently labeled epidermal growth factor (Molecular Probes, Inc.)

In a second embodiment, fluorescently labeled primary or secondary antibodies (Sigma Chemical Co. St. Louis, MO; Molecular Probes, Inc. Eugene, OR; Caltag Antibody Co.) that specifically label endosomal ligands are used to mark the endosomal compartment in cells.

In a third embodiment, endosomes are fluorescently labeled in cells expressing protein chimeras formed by fusing a green fluorescent protein, or mutants thereof, with a receptor whose internalization labels endosomes. Chimeras of the EGF, transferrin, and low density lipoprotein receptors are examples of these molecules.

20

### Lysosome labeling

In one embodiment, membrane permeant lysosome-specific luminescent reagents are used to label the lysosomal compartment of living and fixed cells. These reagents include the luminescent molecules neutral red, N-(3-((2,4-dinitrophenyl)amino)propyl)-N-(3-aminopropyl)methylamine, and the LysoTracker probes which report intralysosomal pH as well as the dynamic distribution of lysosomes (Molecular Probes, Inc.)

In a second embodiment, antibodies against lysosomal antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label lysosomal components that are localized in specific lysosomal domains. Examples of these components are the degradative enzymes involved in cholesterol ester hydrolysis,

30

membrane protein proteases, and nucleases as well as the ATP-driven lysosomal proton pump.

5 In a third embodiment, protein chimeras consisting of a lysosomal protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the lysosomal domain. Examples of these components are the degradative enzymes involved in cholesterol ester hydrolysis, membrane protein proteases, and nucleases as well as the ATP-driven lysosomal proton pump.

#### 10 **Cytoplasmic fluorescence labeling**

In one embodiment, cell permeant fluorescent dyes (Molecular Probes, Inc.) with a reactive group are reacted with living cells. Reactive dyes including monobromobimane, 5-chloromethylfluorescein diacetate, carboxy fluorescein diacetate succinimidyl ester, and chloromethyl tetramethylrhodamine are examples of cell  
15 permeant fluorescent dyes that are used for long term labeling of the cytoplasm of cells.

In a second embodiment, polar tracer molecules such as Lucifer yellow and cascade blue-based fluorescent dyes (Molecular Probes, Inc.) are introduced into cells using bulk loading methods and are also used for cytoplasmic labeling.

20 In a third embodiment, antibodies against cytoplasmic components (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to fluorescently label the cytoplasm. Examples of cytoplasmic antigens are many of the enzymes involved in intermediary metabolism. Enolase, phosphofructokinase, and acetyl-CoA dehydrogenase are examples of uniformly distributed cytoplasmic antigens.

25 In a fourth embodiment, protein chimeras consisting of a cytoplasmic protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the cytoplasm. Fluorescent chimeras of uniformly distributed proteins are used to label the entire cytoplasmic domain. Examples of these proteins are many of the proteins involved in intermediary metabolism and include enolase, lactate dehydrogenase, and hexokinase.

30 In a fifth embodiment, antibodies against cytoplasmic antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label cytoplasmic components that are localized in specific cytoplasmic sub-domains.

Examples of these components are the cytoskeletal proteins actin, tubulin, and cytokeratin. A population of these proteins within cells is assembled into discrete structures, which in this case, are fibrous. Fluorescence labeling of these proteins with antibody-based reagents therefore labels a specific sub-domain of the cytoplasm.

5 In a sixth embodiment, non-antibody-based fluorescently labeled molecules that interact strongly with cytoplasmic proteins are used to label specific cytoplasmic components. One example is a fluorescent analog of the enzyme DNase I (Molecular Probes, Inc.) Fluorescent analogs of this enzyme bind tightly and specifically to cytoplasmic actin, thus labeling a sub-domain of the cytoplasm. In another example,  
10 fluorescent analogs of the mushroom toxin phalloidin or the drug paclitaxel (Molecular Probes, Inc.) are used to label components of the actin- and microtubule-cytoskeletons, respectively.

In a seventh embodiment, protein chimeras consisting of a cytoplasmic protein genetically fused to an intrinsically luminescent protein such as the green fluorescent  
15 protein, or mutants thereof, are used to label specific domains of the cytoplasm. Fluorescent chimeras of highly localized proteins are used to label cytoplasmic sub-domains. Examples of these proteins are many of the proteins involved in regulating the cytoskeleton. They include the structural proteins actin, tubulin, and cytokeratin as well as the regulatory proteins microtubule associated protein 4 and  $\alpha$ -actinin.

#### 20 Nuclear labeling

In one embodiment, membrane permeant nucleic-acid-specific luminescent reagents (Molecular Probes, Inc.) are used to label the nucleus of living and fixed cells. These reagents include cyanine-based dyes (*e.g.*, TOTO<sup>®</sup>, YOYO<sup>®</sup>, and BOBO<sup>™</sup>),  
25 phenanthridines and acridines (*e.g.*, ethidium bromide, propidium iodide, and acridine orange), indoles and imidazoles (*e.g.*, Hoechst 33258, Hoechst 33342, and 4',6-diamidino-2-phenylindole), and other similar reagents (*e.g.*, 7-aminoactinomycin D, hydroxystilbamidine, and the psoralens).

In a second embodiment, antibodies against nuclear antigens (Sigma Chemical  
30 Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label nuclear components that are localized in specific nuclear domains. Examples of these components are the macromolecules involved in maintaining DNA structure and

function. DNA, RNA, histones, DNA polymerase, RNA polymerase, lamins, and nuclear variants of cytoplasmic proteins such as actin are examples of nuclear antigens.

In a third embodiment, protein chimeras consisting of a nuclear protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the nuclear domain. Examples of these proteins are many of the proteins involved in maintaining DNA structure and function. Histones, DNA polymerase, RNA polymerase, lamins, and nuclear variants of cytoplasmic proteins such as actin are examples of nuclear proteins.

#### **Mitochondrial labeling**

In one embodiment, membrane permeant mitochondrial-specific luminescent reagents (Molecular Probes, Inc.) are used to label the mitochondria of living and fixed cells. These reagents include rhodamine 123, tetramethyl rosamine, JC-1, and the MitoTracker reactive dyes.

In a second embodiment, antibodies against mitochondrial antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label mitochondrial components that are localized in specific mitochondrial domains. Examples of these components are the macromolecules involved in maintaining mitochondrial DNA structure and function. DNA, RNA, histones, DNA polymerase, RNA polymerase, and mitochondrial variants of cytoplasmic macromolecules such as mitochondrial tRNA and rRNA are examples mitochondrial antigens. Other examples of mitochondrial antigens are the components of the oxidative phosphorylation system found in the mitochondria (*e.g.*, cytochrome c, cytochrome c oxidase, and succinate dehydrogenase).

In a third embodiment, protein chimeras consisting of a mitochondrial protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the mitochondrial domain. Examples of these components are the macromolecules involved in maintaining mitochondrial DNA structure and function. Examples include histones, DNA polymerase, RNA polymerase, and the components of the oxidative phosphorylation system found in the mitochondria (*e.g.*, cytochrome c, cytochrome c oxidase, and succinate dehydrogenase).

### Endoplasmic reticulum labeling

In one embodiment, membrane permeant endoplasmic reticulum-specific luminescent reagents (Molecular Probes, Inc.) are used to label the endoplasmic reticulum of living and fixed cells. These reagents include short chain carbocyanine dyes (*e.g.*, DiOC<sub>6</sub> and DiOC<sub>3</sub>), long chain carbocyanine dyes (*e.g.*, DiIC<sub>16</sub> and DiIC<sub>18</sub>),  
5 and luminescently labeled lectins such as concanavalin A.

In a second embodiment, antibodies against endoplasmic reticulum antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label endoplasmic reticulum components that are localized in specific endoplasmic reticulum  
10 domains. Examples of these components are the macromolecules involved in the fatty acid elongation systems, glucose-6-phosphatase, and HMG CoA-reductase.

In a third embodiment, protein chimeras consisting of a endoplasmic reticulum protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the endoplasmic reticulum  
15 domain. Examples of these components are the macromolecules involved in the fatty acid elongation systems, glucose-6-phosphatase, and HMG CoA-reductase.

### Golgi labeling

In one embodiment, membrane permeant Golgi-specific luminescent reagents (Molecular Probes, Inc.) are used to label the Golgi of living and fixed cells. These  
20 reagents include luminescently labeled macromolecules such as wheat germ agglutinin and Brefeldin A as well as luminescently labeled ceramide.

In a second embodiment, antibodies against Golgi antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label Golgi components that are localized in specific Golgi domains. Examples of these components are N-  
25 acetylglucosamine phosphotransferase, Golgi-specific phosphodiesterase, and mannose-6-phosphate receptor protein.

In a third embodiment, protein chimeras consisting of a Golgi protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the Golgi domain. Examples of these  
30 components are N-acetylglucosamine phosphotransferase, Golgi-specific phosphodiesterase, and mannose-6-phosphate receptor protein.

While many of the examples presented involve the measurement of single cellular processes, this is again intended for purposes of illustration only. Multiple parameter high-content screens can be produced by combining several single parameter screens into a multiparameter high-content screen or by adding cellular parameters to any existing high-content screen. Furthermore, while each example is described as being based on either live or fixed cells, each high-content screen can be designed to be used with both live and fixed cells.

Those skilled in the art will recognize a wide variety of distinct screens that can be developed based on the disclosure provided herein. There is a large and growing list of known biochemical and molecular processes in cells that involve translocations or reorganizations of specific components within cells. The signaling pathway from the cell surface to target sites within the cell involves the translocation of plasma membrane-associated proteins to the cytoplasm. For example, it is known that one of the src family of protein tyrosine kinases, pp60c-src (Walker et al (1993), *J. Biol. Chem.* 268:19552-19558) translocates from the plasma membrane to the cytoplasm upon stimulation of fibroblasts with platelet-derived growth factor (PDGF). Additionally, the targets for screening can themselves be converted into fluorescence-based reagents that report molecular changes including ligand-binding and post-translocational modifications.

## Example 10. Protease Biosensors

### (1) Background

As used herein, the following terms are defined as follows:

- Reactant – the parent biosensor that interacts with the proteolytic enzyme.
- Product – the signal-containing proteolytic fragment(s) generated by the interaction of the reactant with the enzyme.
- Reactant Target Sequence – an amino acid sequence that imparts a restriction on the cellular distribution of the reactant to a particular subcellular domain of the cell.
- Product Target Sequence – an amino acid sequence that imparts a restriction on the cellular distribution of the signal-containing product(s) of the targeted enzymatic reaction to a particular subcellular domain of the cell. If the product is initially localized within a membrane bound compartment, then the Product Target

Sequence must incorporate the ability to export the product out of the membrane-bound compartment. A bi-functional sequence can be used, which first moves the product out of the membrane-bound compartment, and then targets the product to the final compartment. In general, the same amino acid sequences can act as either  
5 or both reactant target sequences and product target sequences. Exceptions to this include amino acid sequences which target the nuclear envelope, Golgi apparatus, endoplasmic reticulum, and which are involved in farnesylation, which are more suitable as reactant target sequences.

- Protease Recognition Site – an amino acid sequence that imparts specificity by  
10 mimicking the substrate, providing a specific binding and cleavage site for a protease. Although typically a short sequence of amino acids representing the minimal cleavage site for a protease (e.g. DEVD for caspase-3, Villa, P., S.H. Kaufmann, and W.C. Earnshaw. 1997. Caspases and caspase inhibitors. *Trends Biochem Sci.* 22:388-93), greater specificity may be established by using a longer  
15 sequence from an established substrate.

- Compartment – any cellular sub-structure or macromolecular component of the cell, whether it is made of protein, lipid, carbohydrate, or nucleic acid. It could be a macromolecular assembly or an organelle (a membrane delimited cellular component). Compartments include, but are not limited to, cytoplasm, nucleus,  
20 nucleolus, inner and outer surface of nuclear envelope, cytoskeleton, peroxisome, endosome, lysosome, inner leaflet of plasma membrane, outer leaflet of plasma membrane, outer leaflet of mitochondrial membrane, inner leaflet of mitochondrial membrane, Golgi, endoplasmic reticulum, or extracellular space.

Signal – an amino acid sequence that can be detected. This includes, but is not  
25 limited to inherently fluorescent proteins (e.g. Green Fluorescent Protein), cofactor-requiring fluorescent or luminescent proteins (e.g. phycobiliproteins or luciferases), and epitopes recognizable by specific antibodies or other specific natural or unnatural binding probes, including but not limited to dyes, enzyme cofactors and engineered binding molecules, which are fluorescently or luminescently labeled.  
30 Also included are site-specifically labeled proteins that contain a luminescent dye. Methodology for site-specific labeling of proteins includes, but is not limited to, engineered dye-reactive amino acids (Post, et al., *J. Biol. Chem.* 269:12880-12887



(1994)), enzyme-based incorporation of luminescent substrates into proteins (Buckler, et al., *Analyt. Biochem.* 209:20-31 (1993); Takashi, *Biochemistry.* 27:938-943 (1988)), and the incorporation of unnatural labeled amino acids into proteins (Noren, et al., *Science.* 244:182-188 (1989)).

- 5 • Detection – a means for recording the presence, position, or amount of the signal. The approach may be direct, if the signal is inherently fluorescent, or indirect, if, for example, the signal is an epitope that must be subsequently detected with a labeled antibody. Modes of detection include, but are not limited to, the spatial position of fluorescence, luminescence, or phosphorescence: (1) intensity; (2) polarization; (3) lifetime; (4) wavelength; (5) energy transfer; and (6) recovery after photobleaching.

The basic principle of the protease biosensors of the present invention is to spatially separate the reactants from the products generated during a proteolytic reaction. The separation of products from reactants occurs upon proteolytic cleavage of the protease recognition site within the biosensor, allowing the products to bind to, diffuse into, or be imported into compartments of the cell different from those of the reactant. This spatial separation provides a means of quantitating a proteolytic process directly in living or fixed cells. Some designs of the biosensor provide a means of restricting the reactant (uncleaved biosensor) to a particular compartment by a protein sequence ("reactant target sequence") that binds to or imports the biosensor into a compartment of the cell. These compartments include, but are not limited to any cellular substructure, macromolecular cellular component, membrane-limited organelles, or the extracellular space. Given that the characteristics of the proteolytic reaction are related to product concentration divided by the reactant concentration, the spatial separation of products and reactants provides a means of uniquely quantitating products and reactants in single cells, allowing a more direct measure of proteolytic activity.

The molecular-based biosensors may be introduced into cells via transfection and the expressed chimeric proteins analyzed in transient cell populations or stable cell lines. They may also be pre-formed, for example by production in a prokaryotic or eukaryotic expression system, and the purified protein introduced into the cell via a number of physical mechanisms including, but not limited to, micro-injection, scrape loading, electroporation, signal-sequence mediated loading, etc.

Measurement modes may include, but are not limited to, the ratio or difference in fluorescence, luminescence, or phosphorescence: (a) intensity; (b) polarization; or (c) lifetime between reactant and product. These latter modes require appropriate spectroscopic differences between products and reactants. For example, cleaving a reactant containing a limited-mobile signal into a very small translocating component and a relatively large non-translocating component may be detected by polarization. Alternatively, significantly different emission lifetimes between reactants and products allow detection in imaging and non-imaging modes.

One example of a family of enzymes for which this biosensor can be constructed to report activity is the caspases. Caspases are a class of proteins that catalyze proteolytic cleavage of a wide variety of targets during apoptosis. Following initiation of apoptosis, the Class II "downstream" caspases are activated and are the point of no return in the pathway leading to cell death, resulting in cleavage of downstream target proteins. In specific examples, the biosensors described here were engineered to use nuclear translocation of cleaved GFP as a measurable indicator of caspase activation. Additionally, the use of specific recognition sequences that incorporate surrounding amino acids involved in secondary structure formation in naturally occurring proteins may increase the specificity and sensitivity of this class of biosensor.

Another example of a protease class for which this biosensor can be constructed to report activity is zinc metalloproteases. Two specific examples of this class are the biological toxins derived from *Clostridial* species (*C. botulinum* and *C. tetani*) and *Bacillus anthracis*. (Herreros et al. *In The Comprehensive Sourcebook of Bacterial Protein Toxins*. J.E. Alouf and J.H. Freer, Eds., 2<sup>nd</sup> edition, San Diego, Academic Press, 1999; pp 202-228.) These bacteria express and secrete zinc metalloproteases that enter eukaryotic cells and specifically cleave distinct target proteins. For example, the anthrax protease from *Bacillus anthracis* is delivered into the cytoplasm of target cells via an accessory pore-forming protein, where its proteolytic activity inactivates the MAP-kinase signaling cascade through cleavage of mitogen activated protein kinase kinases 1 or 2 (MEK1 or MEK2). (Leppla, S.A. *In The Comprehensive Sourcebook of Bacterial Protein Toxins*. J.E. Alouf and J.H. Freer, Eds., 2<sup>nd</sup> edition, San Diego, Academic Press, 1999; pp243-263.) The toxin biosensors described here take

advantage of the natural subcellular localization of these and other target proteins to achieve reactant targeting. Upon cleavage, the signal (with or without a product target sequence) is separated from the reactant to create a high-content biosensor.

One of skill in the art will recognize that the protein biosensors of this aspect of the invention can be adapted to report the activity of any member of the caspase family of proteases, as well as any other protease, by a substitution of the appropriate protease recognition site in any of the constructs (see Figure 29B). These biosensors can be used in high-content screens to detect in vivo activation of enzymatic activity and to identify specific activity based on cleavage of a known recognition motif. This screen can be used for both live cell and fixed end-point assays, and can be combined with additional measurements to provide a multi-parameter assay.

Thus, in another aspect the present invention provides recombinant nucleic acids encoding a protease biosensor, comprising:

- a. a first nucleic acid sequence that encodes at least one detectable polypeptide signal;
- b. a second nucleic acid sequence that encodes at least one protease recognition site, wherein the second nucleic acid sequence is operatively linked to the first nucleic acid sequence that encodes the at least one detectable polypeptide signal; and
- c. a third nucleic acid sequence that encodes at least one reactant target sequence, wherein the third nucleic acid sequence is operatively linked to the second nucleic acid sequence that encodes the at least one protease recognition site.

In this aspect, the first and third nucleic acid sequences are separated by the second nucleic acid sequence, which encodes the protease recognition site.

In a further embodiment, the recombinant nucleic acid encoding a protease biosensor comprises a fourth nucleic acid sequence that encodes at least one product target sequence, wherein the fourth nucleic acid sequence is operatively linked to the first nucleic acid sequence that encodes the at least one detectable polypeptide signal.

In a further embodiment, the recombinant nucleic acid encoding a protease biosensor comprises a fifth nucleic acid sequence that encodes at least one detectable

polypeptide signal, wherein the fifth nucleic acid sequence is operatively linked to the third nucleic acid sequence that encodes the reactant target sequence.

In a preferred embodiment, the detectable polypeptide signal is selected from the group consisting of fluorescent proteins, luminescent proteins, and sequence epitopes. In a most preferred embodiment, the first nucleic acid encoding a polypeptide sequence comprises a sequence selected from the group consisting of SEQ ID NOS: 35, 37, 39, 41, 43, 45, 47, 49, and 51.

In another preferred embodiment, the second nucleic acid encoding a protease recognition site comprises a sequence selected from the group consisting of SEQ ID NOS: 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, and 121. In another preferred embodiment, the third nucleic acid encoding a reactant target sequence comprises a sequence selected from the group consisting of SEQ ID NOS: 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, and 151.

In a most preferred embodiment, the recombinant nucleic acid encoding a protease biosensor comprises a sequence substantially similar to sequences selected from the group consisting of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33.

In another aspect, the present invention provides a recombinant expression vector comprising nucleic acid control sequences operatively linked to the above-described recombinant nucleic acids. In a still further aspect, the present invention provides genetically engineered host cells that have been transfected with the recombinant expression vectors of the invention.

In another aspect, the present invention provides recombinant protease biosensors comprising

- a. a first domain comprising at least one detectable polypeptide signal;
- b. a second domain comprising at least one protease recognition site; and
- c. a third domain comprising at least one reactant target sequence; wherein the first domain and the third domain are separated by the second domain.

Inherent in this embodiment is the concept that the reactant target sequence restricts the cellular distribution of the reactant, with redistribution of the product occurring after activation (ie: protease cleavage). This redistribution does not require a complete sequestration of products and reactants, as the product distribution can partially overlap the reactant distribution in the absence of a product targeting signal (see below).

In a preferred embodiment, the recombinant protease biosensor further comprises a fourth domain comprising at least one product target sequence, wherein the fourth domain and the first domain are operatively linked and are separated from the third domain by the second domain. In another embodiment, the recombinant protease biosensor further comprises a fifth domain comprising at least one detectable polypeptide signal, wherein the fifth domain and the third domain are operatively linked and are separated from the first domain by the second domain.

In a preferred embodiment, the detectable polypeptide signal domain (first or fifth domain) is selected from the group consisting of fluorescent proteins, luminescent proteins, and sequence epitopes. In a most preferred embodiment, the detectable polypeptide signal domain comprises a sequence selected from the group consisting of SEQ ID NOS:36, 38, 40, 42, 44, 46, 48, 50, and 52.

In another preferred embodiment, the second domain comprising a protease recognition site comprises a sequence selected from the group consisting of SEQ ID NOS:54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122. In another preferred embodiment, the reactant and/or target sequence domains comprise a sequence selected from the group consisting of SEQ ID NOS:124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, and 152.

In a most preferred embodiment, the recombinant protease biosensor comprises a sequence substantially similar to sequences selected from the group consisting of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34.

In a still further embodiment, the present invention provides methods and kits for automated analysis of cells, comprising using cells that possess the protease biosensors of the invention to identify compounds that affect protease activity. The

method can be combined with the other methods of the invention in a variety of possible multi-parametric assays.

In these various embodiments, the basic protease biosensor is composed of multiple domains, including at least a first detectable polypeptide signal domain, at least one reactant target domain, and at least one protease recognition domain, wherein the detectable signal domain and the reactant target domain are separated by the protease recognition domain. Thus, the exact order of the domains in the molecule is not generally critical, so long as the protease recognition domain separates the reactant target and first detectable signal domain. For each domain, one or more one of the specified recognition sequences is present.

In some cases, the order of the domains in the biosensor may be critical for appropriate targeting of product(s) and/or reactant to the appropriate cellular compartment(s). For example, the targeting of products or reactants to the peroxisome requires that the peroxisomal targeting domain comprise the last three amino acids of the protein. Determination of those biosensor in which the relative placement of targeting domains within the biosensor is critical can be determined by one of skill in the art through routine experimentation.

Some examples of the basic organization of domains within the protease biosensor are shown in Figure 30. One of skill in the art will recognize that any one of a wide variety of protease recognition sites, product target sequences, polypeptide signals, and/or product target sequences can be used in various combinations in the protein biosensor of the present invention, by substituting the appropriate coding sequences into the multi-domain construct. Non-limiting examples of such alternative sequences are shown in Figure 29A-29C. Similarly, one of skill in the art will recognize that modifications, substitutions, and deletions can be made to the coding sequences and the amino acid sequence of each individual domain within the biosensor, while retaining the function of the domain. Such various combinations of domains and modifications, substitutions and deletions to individual domains are within the scope of the invention.

As used herein, the term "coding sequence" or a sequence which "encodes" a particular polypeptide sequence, refers to a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro

or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

As used herein, the term DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control sequences need always be present in a recombinant vector so long as the DNA sequence of interest is capable of being transcribed and translated appropriately.

As used herein, the term "operatively linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operatively linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operatively linked" to the coding sequence.

Furthermore, a nucleic acid coding sequence is operatively linked to another nucleic acid coding sequences when the coding region for both nucleic acid molecules are capable of expression in the same reading frame. The nucleic acid sequences need not be contiguous, so long as they are capable of expression in the same reading frame. Thus, for example, intervening coding regions can be present between the specified nucleic acid coding sequences, and the specified nucleic acid coding regions can still be considered "operatively linked".

The intervening coding sequences between the various domains of the biosensors can be of any length so long as the function of each domain is retained.

Generally, this requires that the two-dimensional and three-dimensional structure of the intervening protein sequence does not preclude the binding or interaction requirements of the domains of the biosensor, such as product or reactant targeting, binding of the protease of interest to the biosensor, fluorescence or luminescence of the detectable polypeptide signal, or binding of fluorescently labeled epitope-specific antibodies.

One case where the distance between domains of the protease biosensor is important is where the goal is to create a fluorescence resonance energy transfer pair. In this case, the FRET signal will only exist if the distance between the donor and acceptor is sufficiently small as to allow energy transfer (Tsien, Heim and Cubbit, WO 97/28261). The average distance between the donor and acceptor moieties should be between 1 nm and 10 nm with a preference of between 1 nm and 6 nm. This is the physical distance between donor and acceptor. The intervening sequence length can vary considerably since the three dimensional structure of the peptide will determine the physical distance between donor and acceptor.

"Recombinant expression vector" includes vectors that operatively link a nucleic acid coding region or gene to any promoter capable of effecting expression of the gene product. The promoter sequence used to drive expression of the protease biosensor may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). The expression vector must be replicable in the host organisms either as an episome or by integration into host chromosomal DNA. In a preferred embodiment, the expression vector comprises a plasmid. However, the invention is intended to include any other suitable expression vectors, such as viral vectors.

The phrase "substantially similar" is used herein in reference to the nucleotide sequence of DNA, or the amino acid sequence of protein, having one or more conservative or non-conservative variations from the protease biosensor sequences disclosed herein, including but not limited to deletions, additions, or substitutions wherein the resulting nucleic acid and/or amino acid sequence is functionally equivalent to the sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same protease biosensor as the nucleic acid and amino acid compositions disclosed and



claimed herein. For example, functionally equivalent DNAs encode protease biosensors that are the same as those disclosed herein or that have one or more conservative amino acid variations, such as substitutions of non-polar residues for other non-polar residues or charged residues for similarly charged residues, or addition  
5 to/deletion from regions of the protease biosensor not critical for functionality. These changes include those recognized by those of skill in the art as substitutions, deletions, and/or additions that do not substantially alter the tertiary structure of the protein.

As used herein, substantially similar sequences of nucleotides or amino acids share at least about 70%-75% identity, more preferably 80-85% identity, and most  
10 preferably 90-95% identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology (due to the degeneracy of the genetic code) or that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

The term "heterologous" as it relates to nucleic acid sequences such as coding  
15 sequences and control sequences, denotes sequences that are not normally associated with a region of a recombinant construct, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct is an identifiable segment of nucleic acid within or attached to another nucleic acid molecule  
20 that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence  
25 itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a host cell transformed with a construct which is not normally present in the host cell would be considered heterologous for purposes of this invention.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press),  
30 *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutscher, ed., (1990) Academic Press, Inc.); PCR

*Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2<sup>nd</sup> Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

The biosensors of the present invention are constructed and used to transfect host cells using standard techniques in the molecular biological arts. Any number of such techniques, all of which are within the scope of this invention, can be used to generate protease biosensor-encoding DNA constructs and genetically transfected host cells expressing the biosensors. The non-limiting examples that follow demonstrate one such technique for constructing the biosensors of the invention.

#### EXAMPLE OF PROTEASE BIOSENSOR CONSTRUCTION AND USE:

In the following examples, caspase-specific biosensors with specific product target sequences have been constructed using sets of 4 primers (2 sense and 2 antisense). These primers have overlap regions at their termini, and are used for PCR via a primer walking technique. (Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) The two sense primers were chosen to start from the 5' polylinker (BspI) of the GFP-containing vector (Clontech, California) to the middle of the designed biosensor sequence. The two antisense primers start from a 3' GFP vector site (Bam HI), and overlap with the sense primers by 12 nucleotides in the middle.

PCR conditions were as follows: 94°C for 30 seconds for denaturation, 55°C for 30 seconds for annealing, and 72°C for 30 seconds for extension for 15 cycles. The primers have restriction endonuclease sites at both ends, facilitating subsequent cloning of the resulting PCR product.

The resulting PCR product was gel purified, cleaved at BspE1 and BamH1 restriction sites present in the primers, and the resulting fragment was gel purified. Similarly, the GFP vector (Clontech, San Francisco, CA) was digested at BspE1 and BamH1 sites in the polylinker. Ligation of the GFP vector and the PCR product was performed using standard techniques at 16°C overnight. *E. coli* cells were transfected

with the ligation mixtures using standard techniques. Transformed cells were selected on LB-agar with an appropriate antibiotic.

**Cells and transfections.** For DNA transfection, BHK cells and MCF-7 cells  
5 were cultured to 50-70% confluence in 6 well plates containing 3 ml of minimal Eagle's medium (MEM) with 10% fetal calf serum, 1 mM L-glutamine, 50 µg/ml streptomycin, 50 µg/ml penicillin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 µg/ml of bovine insulin (for MCF-7 cell only) at 37 °C in a 5% CO<sub>2</sub> incubator for about 36 hours. The cells were washed with serum free MEM media and  
10 incubated for 5 hours with 1 ml of transfection mixture containing 1 µg of the appropriate plasmid and 4 µg of lipofectimine (BRL) in the serum free MEM media. Subsequently, the transfection medium was removed and replaced with 3 ml of normal culture media. The transfected cells were maintained in growth medium for at least 16 hours before performing selection of the stable cells based on standard molecular  
15 biology methods (Ausubel. et al 1995).

**Apoptosis assay.** For apoptosis assays, the cells (BHK, MCF-7) stably transfected with the appropriate protease biosensor expression vector were plated on tissue culture treated 96-well plates at 50-60% confluence and cultured overnight at  
20 37°C, 5% CO<sub>2</sub>. Varying concentrations of cis-platin, staurosporine, or paclitaxel in normal culture media were freshly prepared from stock and added to cell culture dishes to replace the old culture media. The cells were then observed with the cell screening system of the present invention at the indicated time points either as live cell experiments or as fixed end-point experiments.

25

#### 1. Construction of 3-domain protease biosensors

##### a. Caspase-3 biosensor with an annexin II reactant targeting domain (pljkGFP).

The design of this biosensor is outlined in Figure 31, and its sequence is shown  
30 in SEQ ID NO:1 and 2.

**Primers for Caspase 3, Product target sequence = none (CP3GFP-CYTO):**

- 1) TCA TCA TCC GGA GCT GGA GCC GGA GCT GGC CGA TCG GCT GTT  
AAA TCT GAA GGA AAG AGA AAG TGT GAC GAA GTT GAT GGA ATT  
5 GAT GAA GTA GCA (SEQ ID NO:153)
- 2) GAA GAA GGA TCC GGC ACT TGG GGG TGT AGA ATG AAC ACC  
CTC CAA GCT GAG CTT GCA CAG GAT TTC GTG GAC AGT AGA  
CAT AGT ACT TGC TAC TTC ATC (SEQ ID NO:154)
- 3) TCA TCA TCC GGA GCT GGA (SEQ ID NO:155)
- 10 4) GAA GAA GGA TCC GGC ACT (SEQ ID NO:156)

This biosensor is restricted to the cytoplasm by the reactant target sequence. The reactant target sequence is the annexin II cytoskeletal binding domain (MSTVHEILCKLSLEGVHSTPPSA) (SEQ ID NO:124) (Figure 29C) (Eberhard et  
15 al. 1997. *Mol. Biol. Cell* 8:293a). The enzyme recognition site corresponds to two copies of the amino acid sequence DEVD (SEQ ID NO:60) (Figure 29B), which serves as the recognition site of caspase-3. Other examples with different numbers of protease recognition sites and/or additional amino acids from a naturally occurring protease recognition site are shown below. The signal domain is EGFP (SEQ ID  
20 NO:46) (Figure 29A) (Clontech, California). The parent biosensor (the reactant) is restricted to the cytoplasm by binding of the annexin II domain to the cytoskeleton, and is therefore excluded from the nucleus. Upon cleavage of the protease recognition site by caspase 3, the signal domain (EGFP) is released from the reactant targeting domain (annexin II), and is distributed throughout the whole volume of the cell, because it lacks  
25 any specific targeting sequence and is small enough to enter the nucleus passively. (Fig 32)

The biosensor response is measured by quantitating the effective cytoplasm-to-nuclear translocation of the signal (see above). Measurement of the response is by one of several modes, including integrated or average nuclear region intensity, the ratio or  
30 difference of the integrated or average cytoplasm intensity to integrated or average nuclear intensity. The nucleus is defined using a DNA-specific dye, such as Hoechst 33342.

This biosensor provides a measure of the proteolytic activity around the annexin II cytoskeleton binding sites within the cell. Given the dispersed nature of the cytoskeleton and the effectively diffuse state of cytosolic enzymes, this provides an effective measure of the cytoplasm in general.

5

### Results & Discussion:

Fig 32 illustrates images before and after stimulation of apoptosis by cis-platin in BHK cells, transfected with the caspase 3 biosensor. The images clearly illustrate accumulation of fluorescence in the nucleus. Generation of the spatial change in fluorescence is non-reversible and thus the timing of the assay is flexible. Controls for this biosensor include using a version in which the caspase-3-specific site has been omitted. In addition, disruption of the cytoskeleton with subsequent cell rounding did not produce the change in fluorescence distribution. Our experiments demonstrate the correlation of nuclear condensation with activation of caspase activity. We have also tested this biosensor in MCF-7 cells. A recent report measured a peak response in caspase-3 activity 6 h after stimulation of MCF-7 cells with etoposide accompanied by cleavage of PARP (Benjamin et al. 1998. *Mol Pharmacol.* 53:446-50). However, another recent report found that MCF-7 cells do not possess caspase-3 activity and, in fact, the caspase-3 gene is functionally deleted (Janicke et al. 1998. *J Biol Chem.* 273:9357-60). Caspase-3 activity was not detected with the caspase biosensor in MCF-7 cells after a 15 h treatment with 100  $\mu$ M etoposide.

Janicke et al., (1998) also indicated that many of the conventional substrates of caspase-3 were cleaved in MCF-7 cells upon treatment with staurosporine. Our experiments demonstrate that caspase activity can be measured using the biosensor in MCF-7 cells when treated with staurosporine. The maximum magnitude of the activation by staurosporine was approximately one-half that demonstrated with cis-platin in BHK cells. This also implies that the current biosensor, although designed to be caspase-3-specific, is indeed specific for a class of caspases rather than uniquely specific for caspase-3. The most likely candidate is caspase-7 (Janicke et al., 1998). These experiments also demonstrated that the biosensor can be used in multiparameter experiments, with the correlation of decreases in mitochondrial membrane potential, nuclear condensation, and caspase activation.

We have specifically tested the effects of paclitaxel on caspase activation using the biosensor. Caspase activity in BHK and MCF-7 cells was stimulated by paclitaxel. It also appears that caspase activation occurred after nuclear morphology changes. One caveat is that, based on the above discussions, the caspase activity reported by the biosensor in this assay is likely to be due to the combination of caspase-3 and, at least, caspase-7 activity.

Consistent with the above results using staurosporine stimulation on MCF-7 cells, paclitaxel also stimulated the activation of caspase activity. The magnitude was similar to that of staurosporine. This experiment used a much narrower range of paclitaxel than previous experiments where nuclear condensation appears to dominate the response.

**b. Caspase biosensor with the microtubule associated protein 4 (MAP4) projection domain (CP8GFPNLS-SIZEPROJ)**

Another approach for restricting the reactant to the cytoplasm is to make the biosensor too large to penetrate the nuclear pores. Cleavage of such a biosensor liberates a product capable of diffusing into the nucleus.

The additional size required for this biosensor is provided by using the projection domain of MAP4 (SEQ ID NO:142) (Figure 29C) (CP8GFPNLS-SIZEPROJ). The projection domain of MAP4 does not interact with microtubules on its own, and, when expressed, is diffusely distributed throughout the cytoplasm, but is excluded from the nucleus due to its size (~120 kD). Thus, this biosensor is distinct from the one using the full length MAP4 sequence. (see below) One of skill in the art will recognize that many other such domains could be substituted for the MAP4 projection domain, including but not limited to multiple copies of any GFP or one or more copies of any other protein that lacks an active NLS and exceeds the maximum size for diffusion into the nucleus (approximately 60 kD; Alberts, B., Bray, D., Raff, M., Roberts, K., Watson, J.D. (Eds.) Molecular Biology of the Cell, third edition, New York: Garland publishing, 1994. pp 561-563). The complete sequence of the resulting biosensor is shown in SEQ ID NO: 3-4. A similar biosensor with a different protease recognition domain is shown in SEQ ID NO:5-6.

**c. Caspase biosensor with a nuclear export signal**

Another approach for restricting the reactant to the cytoplasm is to actively restrict the reactant from the nucleus by using a nuclear export signal. Cleavage of such a biosensor liberates a product capable of diffusing into the nucleus.

5 The *Bacillus anthracis* bacterium expresses a zinc metalloprotease protein complex called anthrax protease. Human mitogen activated protein kinase kinase 1 (MEK 1) (Seger et al., J. Biol. Chem. 267:25628-25631, 1992) possesses an anthrax protease recognition site (amino acids 1-13) (SEQ ID NO:102) (Figure 29B) that is cleaved after amino acid 8, as well as a nuclear export signal at amino acids 32-44  
10 (SEQ ID NO:140) (Figure 29C). Human MEK 2 (Zheng and Guan, J. Biol. Chem. 268:11435-11439, 1993) possesses an anthrax protease recognition site comprising amino acid residues 1-16 (SEQ ID NO:104) (Figure 29B) and a nuclear export signal at amino acids 36-48. (SEQ ID NO:148) (Figure 29C).

The anthrax protease biosensor comprises Fret25 (SEQ ID NO:48) (Figure  
15 29A) as the signal, the anthrax protease recognition site, and the nuclear export signal from MEK 1 or MEK2. (SEQ ID NOS: 7-8 (MEK1); 9-10 (MEK2)) The intact biosensor will be retained in the cytoplasm by virtue of this nuclear export signal (eg., the reactant target site). Upon cleavage of the fusion protein by anthrax protease, the NES will be separated from the GFP allowing the GFP to diffuse into the nucleus.

20

**2. Construction of 4- and 5-domain biosensors**

For all of the examples presented above for 3-domain protease biosensors, a product targeting sequence, including but not limited to those in Figure 29C, such as a nuclear localization sequence (NLS), can be operatively linked to the signal sequence,  
25 and thus cause the signal sequence to segregate from the reactant target domain after proteolytic cleavage. Addition of a second detectable signal domain, including but not limited to those in Figure 29A, operatively linked with the reactant target domain is also useful in allowing measurement of the reaction by multiple means. Specific examples of such biosensors are presented below.

30

**a. 4 domain biosensors**

**1. Caspase biosensors with nuclear localization sequences**

(pcas3nlsGFP; CP3GFPNLS-CYTO):

The design of the biosensor is outlined in Figure 33, and its sequence is shown in SEQ ID NO:11-12. PCR and cloning procedures were performed as described above, except that the following oligonucleotides were used:

5 Primers for Caspase 3, Product target sequence = NLS (CP3GFPNLS-CYTO) :

1) TCA TCA TCC GGA AGA AGG AAA CGA CAA AAG CGA TCG GCT  
GTT AAA TCT GAA GGA AAG AGA AAG TGT GAC GAA GTT GAT GGA  
ATT GAT GAA GTA GCA (SEQ ID NO:157)

10 2) GAA GAA GGA TCC GGC ACT TGG GGG TGT AGA ATG AAC ACC  
CTC CAA GCT GAG CTT GCA CAG GAT TTC GTG GAC AGT AGA  
CAT AGT ACT TGC TAC TTC ATC (SEQ ID NO:154)

3) TCA TCA TCC GGA AGA AGG (SEQ ID NO:158)

4) GAA GAA GGA TCC GGC ACT (SEQ ID NO:156)

15

This biosensor is similar to that shown in SEQ ID NO:2 except upon recognition and cleavage of the protease recognition site, the product is released and the signal accumulates specifically in the nucleus due to the presence of a nuclear localization sequence, RRKRQK (SEQ ID NO:128) (Figure 29C)(Briggs et al., J. Biol. Chem. 273:22745, 1998) attached to the signal. A specific benefit of this construct is that the products are clearly separated from the reactants. The reactants remain in the cytoplasm, while the product of the enzymatic reaction is restricted to the nuclear compartment. The response is measured by quantitating the effective cytoplasm-to-nuclear translocation of the signal, as described above.

25 With the presence of both product and reactant targeting sequences in the parent biosensor, the reactant target sequence should be dominant prior to activation (e.g., protease cleavage) of the biosensor. One way to accomplish this is by masking the product targeting sequence in the parent biosensor until after protease cleavage. In one such example, the product target sequence is functional only when relatively near the end of a polypeptide chain (ie: after protease cleavage). Alternatively, the biosensor may be designed so that its tertiary structure masks the function of the target sequence until after protease cleavage. Both of these approaches include comparing targeting

30



sequences with different relative strengths for targeting. Using the example of the nuclear localization sequence (NLS) and annexin II sequences, different strengths of NLS have been tried with clone selection based on cytoplasmic restriction of the parent biosensor. Upon activation, the product targeting sequence will naturally dominate the  
 5 localization of its associated detectable sequence domain because it is then separated from the reactant targeting sequence.

An added benefit of using this biosensor is that the product is targeted, and thus concentrated, into a smaller region of the cell. Thus, smaller amounts of product are detectable due to the increased concentration of the product. This concentration effect  
 10 is relatively insensitive to the cellular concentration of the reactant. The signal-to-noise ratio (SNR) of such a measurement is improved over the more dispersed distribution of biosensor #1.

Similar biosensors that incorporate either the caspase 6 (SEQ ID NO:66) (Figure 29B) or the caspase 8 protease recognition sequence (SEQ ID NO:74) (Figure  
 15 29B) can be made using the methods described above, but using the following primer sets:

**Primers for Caspase 6, Product target sequence = NLS (CP6GFPNLS-CYTO)**

- 1) TCA TCA TCC GGA AGA AGG AAA CGA CAA AAG CGA TCG  
 20 ACA AGA CTT GTT GAA ATT GAC AAC (SEQ ID NO:159)
- 2) GAA GAA GGA TCC GGC ACT TGG GGG TGT AGA ATG AAC  
 ACC CTC CAA GCT GAG CTT GCA CAG GAT TTC GTG GAC  
 AGT AGA CAT AGT ACT GTT GTC AAT TTC (SEQ ID NO:160)

- 25 3) TCA TCA TCC GGA AGA AGG (SEQ ID NO:158)

- 4) GAA GAA GGA TCC GGC ACT (SEQ ID NO:156)

**Primers for Caspase 8, Product target sequence = NLS (CP8GFPNLS-CYTO)**

- 1) TCA TCA TCC GGA AGA AGG AAA CGA CAA AAG CGA TCG  
 30 TAT CAA AAA GGA ATA CCA GTT GAA ACA GAC AGC GAA GAG  
 CAA CCT TAT (SEQ ID NO:161)
- 2) GAA GAA GGA TCC GGC ACT TGG GGG TGT AGA ATG AAC ACC CTC

CAA GCT GAG CTT GCA CAG GAT TTC GTG GAC AGT AGA CAT AGT  
 ACT ATA AGG TTG CTC (SEQ ID NO:162)

3) TCA TCA TCC GGA AGA AGG (SEQ ID NO:158)

4) GAA GAA GGA TCC GGC ACT (SEQ ID NO:156)

5

The sequence of the resulting biosensors is shown in SEQ ID NO:13-14 (Caspase 6) and SEQ ID NO: 15-16 (Caspase 8). Furthermore, multiple copies of the protease recognition sites can be inserted into the biosensor, yielding the biosensors shown in SEQ ID NO: 17-18 (Caspase 3) and SEQ ID NO:19-20 (Caspase 8).

10

## 2. Caspase 3 biosensor with a second signal domain

An alternative embodiment employs a second signal domain operatively linked to the reactant target domain. In this example, full length MAP4 serves as the reactant target sequence. Upon recognition and cleavage, one product of the reaction, containing the reactant target sequence, remains bound to microtubules in the cytoplasm with its own unique signal, while the other product, containing the product target sequence, diffuses into the nucleus. This biosensor provides a means to measure two activities at once: caspase 3 activity using a translocation of GFP into the nucleus and microtubule cytoskeleton integrity in response to signaling cascades initiated during apoptosis, monitored by the MAP4 reactant target sequence.

20

The basic premise for this biosensor is that the reactant is tethered to the microtubule cytoskeleton by virtue of the reactant target sequence comprising the full length microtubule associated protein MAP4 (SEQ ID NO:152) (Figure 29C). In this case, a DEVD (SEQ ID NO:60) (Figure 29B) recognition motif is located between the EYFP signal (SEQ ID NO:44) (Figure 29A) operatively linked to the reactant target sequence, as well as the EBFP signal (SEQ ID NO:48) (Figure 29A) operatively linked to the C-terminus of MAP4. The resulting biosensor is shown in SEQ ID NO:21-22.

25

This biosensor can also include a product targeting domain, such as an NLS, operatively linked to the signal domain.

30

With this biosensor, caspase-3 cleavage still releases the N-terminal GFP, which undergoes translocation to the nucleus (directed there by the NLS). Also, the MAP4

fragment, which is still intact following proteolysis by caspase-3, continues to report on the integrity of the microtubule cytoskeleton during the process of apoptosis via the second GFP molecule fused to the C-terminus of the biosensor. Therefore, this single chimeric protein allows simultaneous analysis of caspase-3 activity and the polymerization state of the microtubule cytoskeleton during apoptosis induced by a variety of agents. This biosensor is also useful for analysis of potential drug candidates that specifically target the microtubule cytoskeleton, since one can determine whether a particular drug induced apoptosis in addition to affecting microtubules.

This biosensor potentially combines a unique signal for the reactant, fluorescence resonance energy transfer (FRET) from signal 2 to signal 1, and a unique signal localization for the product, nuclear accumulation of signal 1. The amount of product generated will also be indicated by the magnitude of the loss in FRET, but this will be a smaller SNR than the combination of FRET detection of reactant and spatial localization of the product.

FRET can occur when the emission spectrum of a donor overlaps significantly the absorption spectrum of an acceptor molecule. (dos Remedios, C.G., and P.D. Moens. 1995. Fluorescence resonance energy transfer spectroscopy is a reliable "ruler" for measuring structural changes in proteins. Dispelling the problem of the unknown orientation factor. *J Struct Biol.* 115:175-85; Emmanouilidou, E., A.G. Teschemacher, A.E. Pouli, L.I. Nicholls, E.P. Seward, and G.A. Rutter. 1999. Imaging Ca(2+) concentration changes at the secretory vesicle surface with a recombinant targeted cameleon. *Curr Biol.* 9:915-918.) The average physical distance between the donor and acceptor molecules should be between 1 nm and 10 nm with a preference of between 1 nm and 6 nm. The intervening sequence length can vary considerably since the three dimensional structure of the peptide will determine the physical distance between donor and acceptor. This FRET signal can be measured as (1) the amount of quenching of the donor in the presence of the acceptor, (2) the amount of acceptor emission when exciting the donor, and/or (3) the ratio between the donor and acceptor emission. Alternatively, fluorescent lifetimes of donor and acceptor could be measured.

This case adds value to the above FRET biosensor by nature of the existence of the reactant targeting sequence. This sequence allows the placement of the biosensor

into specific compartments of the cell for a more direct readout of activity in those compartments such as the inner surface of the plasma membrane.

The cytoplasmic second signal represents both original reactant plus one part of the product. The nuclear first signal represents another product of the reaction. Thus the enzymatic reaction has the added flexibility in that it can be represented as (1) nuclear  
5 intensity; (2) the nucleus /cytoplasm ratio; (3) the nucleus /cytoplasm FRET ratio; (4) cytoplasmic /cytoplasmic FRET ratio.

The present FRET biosensor design differs from previous FRET-based biosensors (see WO 97/28261; WO9837226) in that its signal measurement is based on  
10 spatial position rather than intensity. The products of the reaction are segregated from the reactants. It is this change in spatial position that is measured. The FRET-based biosensor is based on the separation, but not to another compartment, of a donor and acceptor pair. The intensity change is due to the physical separation of the donor and acceptor upon proteolytic cleavage. The disadvantages of FRET-based biosensors are  
15 (1) the SNR is rather low and difficult to measure, (2) the signal is not fixable. It must be recorded using living cells. Chemical fixation, for example with formaldehyde, cannot preserve both the parent and resultant signal; (3) the range of wavelengths are limiting and cover a larger range of the spectrum due to the presence of two fluorophores or a fluorophore and chromophore; (4) the construction has greater  
20 limitations in that the donor and acceptor must be precisely arranged to ensure that the distance falls within 1-10 nm.

Benefits of the positional biosensor includes: (1) ability to concentrate the signal in order to achieve a higher SNR. (2) ability to be used with either living or fixed cells; (3) only a single fluorescent signal is needed; (4) the arrangement of the domains  
25 of the biosensor is more flexible. The only limiting factor in the application of the positional biosensor is the need to define the spatial position of the signal which requires an imaging method with sufficient spatial resolution to resolve the difference between the reactant compartment and the product compartment.

One of skill in the art will recognize that this approach can be adapted to report  
30 any desired combination of activities by simply making the appropriate substitutions for the protease recognition sequence and the reactant target sequence, including but not limited to those sequences shown in Figure 29A-C.

### 3. Caspase 8 biosensor with a nucleolar localization domain (CP8GFPNUC-CYTO)

This approach (diagrammed in Figure 34) utilizes a biosensor for the detection of caspase-8 activity. In this biosensor, a nucleolar localization signal (RKRIPTYLKSCRRMKRSGFEMSRPIPSHLT) (SEQ ID NO:130) (Figure 29C) (Ueki et al., Biochem. Biophys. Res. Comm. 252:97-100, 1998) was used as the product target sequence, and made by PCR using the primers described below. The PCR product was digested with BspE1 and Pvu1 and gel purified. The vector and the PCR product were ligated as described above.

#### Primers for Caspase 8, Nucleolar localization signal (CP8GFPNUC-CYTO):

- 1) TCA TCA TCC GGA AGA AAA CGT ATA CGT ACT TAC CTC AAG  
TCC TGC AGG CGG ATG AAA AGA (SEQ ID NO:163)
- 2) GAA GAA CGA TCG AGT AAG GTG GGA AGG AAT AGG TCG AGA  
CAT CTC AAA ACC ACT TCT TTT CAT (SEQ ID NO:164)
- 3) TCA TCA TCC GGA AGA AAA (SEQ ID NO:165)
- 4) GAA GAA CGA TCG AGT AAG (SEQ ID NO:166)

The sequence of the resulting biosensor is shown in SEQ ID NO: 23-24. This biosensor includes the protease recognition site for caspase-8 (SEQ ID NO:74) (Figure 29B). A similar biosensor utilizes the protease recognition site for caspase-3. (SEQ ID NO:25-26)

These biosensors could be used with other biosensors that possess the same product signal color that are targeted to separate compartments, such as CP3GFPNLS-CYTO. The products of each biosensor reaction can be uniquely measured due to separation of the products based on the product targeting sequences. Both products from CP8GFPNUC-CYTO and CP3GFPNLS-CYTO are separable due to the different spatial positions, nucleus vs. nucleolus, even though the colors of the products are exactly the same. Assessing the non-nucleolar, nuclear region in order to avoid the spatial overlap of the two signals would perform the measurement of CP3GFPNLS in

the presence of CP8GFPNUC. The loss of the nucleolar region from the nuclear signal is insignificant and does not significantly affect the SNR. The principle of assessing multiple parameters using the same product color significantly expands the number of parameters that can be assessed simultaneously in living cells. This concept can be extended to other non-overlapping product target compartments.

Measurement of translocation to the nucleolar compartment is performed by (1) defining a mask corresponding to the nucleolus based on a nucleolus-specific marker, including but not limited to an antibody to nucleolin (Lischwe et al., 1981. *Exp. Cell Res.* 136:101-109); (2) defining a mask for the reactant target compartment, and (3) determining the relative distribution of the signal between these two compartments. This relative distribution could be represented by the difference in the two intensities or, preferably, the ratio of the intensities between compartments.

The combination of multiple positional biosensors can be complicated if the reactant compartments are overlapping. Although each signal could be measured by simply determining the amount of signal in each product target compartment, higher SNR will be possible if each reactant is uniquely identified and quantitated. This higher SNR can be maximized by adding a second signal domain of contrasting fluorescent property. This second signal may be produced by a signal domain operatively linked to the product targeting sequence, or by FRET (see above), or by a reactant targeting sequence uniquely identifying it within the reactant compartment based on color, spatial position, or fluorescent property including but not limited to polarization or lifetime. Alternatively, for large compartments, such as the cytoplasm, it is possible to place different, same colored biosensors in different parts of the same compartment.

#### **4. Protease biosensors with multiple copies of a second signal domain serving as a reactant target domain**

In another example, (CP8YFPNLS-SIZECFPn) increasing the size of the reactant is accomplished by using multiple inserts of a second signal sequence, for example, ECFP (SEQ ID NO:50) (Figure 29A) (Tsien, R.Y. 1998. *Annu Rev Biochem.* 67:509-44). Thus, the multiple copies of the second signal sequence serve as the reactant target domain by excluding the ability of the biosensor to diffuse into the nucleus. This type of biosensor provides the added benefit of additional signal being

available per biosensor molecule. Aggregation of multiple fluorescent probes also can result in unique signals being manifested, such as FRET, self quenching, excimer formation, etc. This could provide a unique signal to the reactants.

5           **5. Tetanus/botulinum biosensor with trans-membrane targeting domain**

In an alternative embodiment, a trans-membrane targeting sequence is used to tether the reactant to cytoplasmic vesicles, and an alternative protease recognition site is used. The tetanus/botulinum biosensor (SEQ ID NOS:27-28 (cellubrevin); 29-30 (synaptobrevin) consists of an NLS (SEQ ID NO:128) (Figure 29C), Fret25' signal domain (SEQ ID NO:52) (Figure 29A), a tetanus or botulinum zinc metalloprotease recognition site from cellubrevin (SEQ ID NO:106) (Figure 29B) (McMahon et al., Nature 364:346-349, 1993; Martin et al., J. Cell Biol., in press) or synaptobrevin (SEQ ID NO:108) (Figure 29B) (GenBank Accession #U64520), and a trans-membrane sequence from cellubrevin (SEQ ID NO:146) (Figure 29C) or synaptobrevin (SEQ ID NO:144) (Figure 29C) at the 3'-end which tethers the biosensor to cellular vesicles. The N-terminus of each protein is oriented towards the cytoplasm. In the intact biosensor, GFP is tethered to the vesicles. Upon cleavage by the tetanus or botulinum zinc metalloprotease, GFP will no longer be associated with the vesicle and is free to diffuse throughout the cytoplasm and the nucleus.

**b. 5-domain biosensors**

**1. Caspase 3 biosensor with a nuclear localization domain and a second signal domain operatively linked to an annexin II domain**

25           The design of this biosensor is outlined in Figure 35, and the sequence is shown in SEQ ID NO:33-34. This biosensor differs from SEQ ID NO 11-12 by including a second detectable signal, ECFP (SEQ ID NO:50) (Figure 29A) (signal 2) operatively linked to the reactant target sequence.

30           **2. Caspase 3 biosensor with a nuclear localization sequence and a second signal domain operatively linked to a MAP4 projection domain (CP3YFPNLS-CFPCYTO)**

In this biosensor (SEQ ID NO:31-32), an NLS product targeting domain (SEQ ID NO:128) (Figure 29C) is present upstream of an EYFP signal domain (SEQ ID NO:44) (Figure 29A). A DEVD protease recognition domain (SEQ ID NO:60) (Figure 29B) is between after the EYFP signal domain and before the MAP4 projection domain (SEQ ID NO:142) (Figure 29C).

#### Example 11. Fluorescent Biosensor Toxin Characterization

As used herein, "toxin" refers to any organism, macromolecule, or organic or inorganic molecule or ion that alters normal physiological processes found within a cell, or any organism, macromolecule, or organic or inorganic molecule or ion that alters the physiological response to modulators of known physiological processes. Thus, a toxin can mimic a normal cell stimulus, or can alter a response to a normal cell stimulus.

Living cells are the targets of toxic agents that can comprise organisms, macromolecules, or organic or inorganic molecules. A cell-based approach to toxin detection, classification, and identification would exploit the sensitive and specific molecular detection and amplification system developed by cells to sense minute changes in their external milieu. By combining the evolved sensing capability of cells with the luminescent reporter molecules and assays described herein, intracellular molecular and chemical events caused by toxic agents can be converted into detectable spatial and temporal luminescent signals.

When a toxin interacts with a cell, whether it is at the cell surface or within a specific intracellular compartment, the toxin invariably undermines one or more components of the molecular pathways active within the cell. Because the cell is comprised of complex networks of interconnected molecular pathways, the effects of a toxin will likely be transmitted throughout many cellular pathways. Therefore, our strategy is to use molecular markers within key pathways likely to be affected by toxins, including but not limited to cell stress pathways, metabolic pathways, signaling pathways, and growth and division pathways.

We have developed and characterized three classes of cell based luminescent reporter molecules to serve as reporters of toxic threat agents. These 3 classes are as follows:



(1) *Detectors*: general cell stress detection of a toxin;

(2) *Classifiers*: perturbation of key molecular pathway(s) for detection and classification of a toxin; and

(3) *Identifiers*: activity mediated detection and identification of a toxin or a  
5 group of toxins.

Thus, in another aspect of the present invention, living cells are used as biosensors to interrogate the environment for the presence of toxic agents. In one embodiment of this aspect, an automated method for cell based toxin characterization is disclosed that comprises providing an array of locations containing cells to be treated  
10 with a test substance, wherein the cells possess at least a first luminescent reporter molecule comprising a detector and a second luminescent reporter molecule selected from the group consisting of a classifier or an identifier; contacting the cells with the test substance either before or after possession of the first and second luminescent reporter molecules by the cells; imaging or scanning multiple cells in each of the  
15 locations containing multiple cells to obtain luminescent signals from the detector; converting the luminescent signals from the detector into digital data to automatically measure changes in the localization, distribution, or activity of the detector on or in the cell, which indicates the presence of a toxin in the test substance; selectively imaging or scanning the locations containing cells that were contacted with test sample indicated to  
20 have a toxin in it to obtain luminescent signals from the second reporter molecule; converting the luminescent signals from the second luminescent reporter molecule into digital data to automatically measure changes in the localization, distribution, or activity of the classifier or identifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the classifier identifies a cell pathway  
25 that is perturbed by the toxin present in the test substance, or wherein a change in the localization, distribution, structure or activity of the identifier identifies the specific toxin that is present in the test substance. In a preferred embodiment, the cells possess at least a detector, a classifier, and an identifier. In a further preferred embodiment, the digital data derived from the classifier is used to determine which identifier(s) to  
30 employ for identifying the specific toxin or group of toxins.

As used herein, the phrase "the cells possess one or more luminescent reporter molecules" means that the luminescent reporter molecule may be expressed as a

luminescent reporter molecule by the cells, added to the cells as a luminescent reporter molecule, or luminescently labeled by contacting the cell with a luminescently labeled molecule that binds to the reporter molecule, such as a dye or antibody, that binds to the reporter molecule. The luminescent reporter molecule can be expressed or added to the cell either before or after treatment with the test substance.

The luminescent reporters comprising detectors, classifiers, and identifiers may also be distributed separately into single or multiple cell types. For example, one cell type may contain a toxin detector, which, when activated by toxic activity, implies to the user that the same toxin sample should be screened with reporters of the classifier or identifier type in yet another population of cells identical to or different from the cells containing the toxin detector.

The detector, classifier, and identifier can comprise the same reporter molecule, or they can comprise different reporters.

Screening for changes in the localization, distribution, structure or activity of the detectors, classifiers, and/or identifiers can be carried out in either a high throughput or a high content mode. In general, a high-content assay can be converted to a high-throughput assay if the spatial information rendered by the high-content assay can be recoded in such a way as to no longer require optical spatial resolution on the cellular or subcellular levels. For example, a high-content assay for microtubule reorganization can be carried out by optically resolving luminescently labeled cellular microtubules and measuring their morphology (e.g., bundled vs. non-bundled or normal). A high-throughput version of a microtubule reorganization assay would involve only a measurement of total microtubule polymer mass after cellular extraction with a detergent. That is, destabilized microtubules, being more easily extracted, would result in a lower total microtubule mass luminescence signal than unperturbed or drug-stabilized luminescently labeled microtubules in another treated cell population. The luminescent signal emanating from a domain containing one or more cells will therefore be proportional to the total microtubule mass remaining in the cells after toxin treatment and detergent extraction.

The methods for detecting, classifying, and identifying toxins can utilize the same screening methods described throughout the instant application, including but not limited to detecting changes in cytoplasm to nucleus translocation, nucleus or nucleolus

to cytoplasm translocation, receptor internalization, mitochondrial membrane potential, signal intensity, the spectral response of the reporter molecule, phosphorylation, intracellular free ion concentration, cell size, cell shape, cytoskeleton organization, metabolic processes, cell motility, cell substrate attachment, cell cycle events, and organellar structure and function.

In all of these embodiments, the methods can be operated in both toxin-mimetic and toxin-inhibitory modes.

Such techniques to assess the presence of toxins are useful for methods including, but not limited to, monitoring the presence of environmental toxins in test samples and for toxins utilized in chemical and biological weapons; and for detecting the presence and characteristics of toxins during environmental remediation, drug discovery, clinical applications, and during the normal development and manufacturing process by virtually any type of industry, including but not limited to agriculture, food processing, automobile, electronic, textile, medical device, and petroleum industries.

We have developed and characterized examples of luminescent cell-based reporters, distributed across the 3 sensor classes. The methods disclosed herein can be utilized in conjunction with computer databases, and data management, mining, retrieval, and display methods to extract meaningful patterns from the enormous data set generated by each individual reporter or a combinatorial of reporters in response to toxic agents. Such databases and bioinformatics methods include, but are not limited to, those disclosed in U.S. Patent Application Nos. 09/437,976, filed November 10, 1999; 60/145,770 filed July 27, 1999 and U.S. Patent Application Serial No. to be assigned, filed February 19, 2000. (98,068-C)

Any cell type can be used to carry out this aspect of the invention, including prokaryotes such as bacteria and archaeobacteria, and eukaryotes, such as single celled fungi (for example, yeast), molds (for example, Dictyostelium), and protozoa (for example, Euglena). Higher eukaryotes, including, but not limited to, avian, amphibian, insect, and mammalian cells can also be used.

### Examples of Biosensors

Number	Name	Class	Cell Types	Response to model toxins
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				Positive	Negative
1	Mitochondrial Potential [Donnan Equilibrium Dye]	D	<ul style="list-style-type: none"> <li>• LLCPK (pig epithelia)</li> <li>• Rat primary hepatocytes</li> </ul>	Valinomycin (10 nM-100 $\mu$ M) FCCP (10 nM-100 $\mu$ M)	Oligomycin (10 nM)
2	Heat Shock Protein (Hsp 27, Hsp 70) GFP-chimera	D	<ul style="list-style-type: none"> <li>• HeLa</li> <li>• 3T3</li> </ul>	Cadmium (10mM)	TNF- $\alpha$ (100ng/ml)
3	Tubulin-cytoskeleton [ $\beta$ -tubulin-GFP chimera]	C	<ul style="list-style-type: none"> <li>• BHK</li> <li>• HeLa</li> <li>• LLCPK</li> </ul>	Paclitaxel (10 nM-20 $\mu$ M) Curacin-A (5 nM-10 $\mu$ M) Nocadazole (7 nM-12 $\mu$ M) Colchicine (5 nM-10 $\mu$ M) Vinblastine (5 nM-10 $\mu$ M)	Staurosporine (1 nM-1 $\mu$ M)
4	pp38 MAPK- stress signaling [antibody and GFP-chimera]	C	<ul style="list-style-type: none"> <li>• 3T3</li> <li>• LLCPK</li> </ul>	Anisomycin (100 $\mu$ M) Cadmium (10 $\mu$ M)	TNF- $\alpha$ (100 ng/ml)
5	NF- $\kappa$ B- stress signaling [antibody and GFP-chimera]	C	<ul style="list-style-type: none"> <li>• HeLa</li> <li>• 3T3</li> <li>• BHK</li> <li>• SNB19</li> <li>• HepG2</li> <li>• LLCPK</li> </ul>	TNF- $\alpha$ (100ng/ml-0.38pg/ml) IL-1 (4ng/ml-.095pg/ml) Nisin (2-1000 $\mu$ g/ml) Streptolysin (10 $\mu$ g/ml) Anisomycin (100 $\mu$ M)	Anisomycin (10 nM-10 $\mu$ M) Cadmium (1-10 $\mu$ M) Penitrem A (10 $\mu$ M) Valinomycin (1 $\mu$ M)
6	I $\kappa$ B [complement to NF- $\kappa$ B]	C	In many cell types		
7	Tetanus Toxin [Protease activity-based sensor]	I	In many cell types		
8	Anthrax LF [Protease activity-based sensor]	I	In many cell types		

Sensor Class: D= Detector of toxins; C= Classifier of toxins; I= Identifier of toxin or group of toxins  
 The model toxins can generally be purchased from Sigma Chemical Company (St. Louis, MO)

- 5 **Examples of Detectors:** This class of sensors provides a first line signal that indicates the presence of a toxic agent. This class of sensors provides detection of general cellular stress that requires resolution limited only to the domain over which the measurement is being made, and they are amenable to high content screens as well. Thus, either high throughput or high content screening modes may be used, including
- 10 but not limited to translocation of heat shock factors from the cytoplasm to the nucleus,

and changes in mitochondrial membrane potential, intracellular free ion concentration detection (for example,  $\text{Ca}^{2+}$ ;  $\text{H}^+$ ), general metabolic status, cell cycle timing events, and organellar structure and function.

5    1.    Mitochondrial Potential

A key to maintenance of cellular homeostasis is a constant ATP energy charge. The cycling of ATP and its metabolites ADP, AMP, inorganic phosphate, and solution-phase protons is continuously adjusted to meet the catabolic and anabolic needs of the cell. Mitochondria are primarily responsible for maintaining a constant energy charge  
10 throughout the entire cell. To produce ATP from its constituents, mitochondria must maintain a constant membrane potential within the organelle itself. Therefore, measurement of this electrical potential with specific luminescent probes provides a sensitive and rapid readout of cellular stress.

We have utilized a positively charged cyanine dye, JC-1 (Molecular Probes,  
15 Eugene, OR), which diffuses into the cell and readily partitions into the mitochondrial membrane, for measurement of mitochondrial potential. The photophysics of JC-1 are such that when the probe partitions into the mitochondrial membrane and it experiences an electrical potential  $>140$  mV, the probe aggregates and its spectral response is shifted to the red. At membrane potential values  $<140$  mV, JC-1 is primarily  
20 monomeric and its spectral response is shifted toward the blue. Therefore, the ratio of two emission wavelengths (645 nm and 530 nm) of JC-1 partitioned into mitochondria provides a sensitive and continuous measure of mitochondrial membrane potential.

We have been making live cell measurements in a high throughput mode as the basis of a generalized indicator of toxic stress. The goal of our initial experiments was  
25 to determine the ratio of J-aggregates of JC-1 dye to its monomeric form both before and after toxic stress.

**Procedure**

1. Cells were plated and cultured up to overnight.
2. Cells were stained with JC-1 (10  $\mu\text{g}/\text{ml}$ ) for 30 minutes at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator.
- 30 3. Cells were then washed quickly with HBSS at  $37^\circ\text{C}$  (2 times, 150  $\mu\text{l}/\text{well}$ ), the toxins were added if required, and the entire plate was scanned in a plate reader. The JC-1 monomer was measured optimally with a 485 nm excitation/530 nm emission wavelength filter set, and the aggregates were best measured with a 590 nm excitation/645 nm emission wavelength set.

35

## Results

The mitochondrial potential within several types of living cells, and the effects of toxins on the potential were measured using the fluorescence ratio Em 645 (590)/  
5 Em 530 (485) (excitation wavelengths in parentheses). For example, we measured the effect of 10  $\mu$ M valinomycin on the mitochondrial potential within LLCPK cells (pig epithelia). Within seconds of treatment, the toxin induced a more rapid and higher magnitude decrease (an approximately 50% reduction) in mitochondrial potential than that found in untreated cells. Hepatocytes were also determined to be sensitive to  
10 valinomycin, and the changes in mitochondrial potential were nearly complete within seconds to minutes after addition of various concentrations of the toxin.

These results are consistent with mitochondrial potential being a model intracellular detector of cell stress. Because these measurements require no spatial resolution within individual cells, mitochondrial potential measurements can be made  
15 rapidly on an entire cell array (e.g. high throughput). This means, for example, that complex arrays of many cell types can be probed simultaneously and continuously as a generalized toxic response. Such an indicator can provide a first line signal to indicate that a general toxic stress is present in a sample. Further assays can then be conducted to more specifically identify the toxin using cells classifier or identifier type reporter  
20 molecules.

### 2. Heat Shock Proteins

Most mammalian cells will respond to a variety of environmental stimuli with the induction of a family of proteins called stress proteins. Anoxia, amino acid  
25 analogues, sulfhydryl-reacting reagents, transition metal ions, decouplers of oxidative phosphorylation, viral infections, ethanol, antibiotics, ionophores, non-steroidal antiinflammatory drugs, thermal stress and metal chelators are all inducers of cell stress protein synthesis, function, or both. Upon induction, cell stress proteins play a role in folding and unfolding proteins, stabilizing proteins in abnormal configurations, and  
30 repairing DNA damage.

There is evidence that at least four heat shock proteins translocate from the cytoplasm to the nucleus upon stress activation of the cell. These proteins include the

heat shock proteins HSP27 and HSP70, the heat shock cognate HSC70, and the heat shock transcription factor HSF1. Therefore, measurement of cytoplasm to nuclear translocation of these proteins (and other stress proteins that translocate from the cytoplasm to the nucleus upon a cell stress) will provide a rapid readout of cellular stress.

We have tested the response of an HSP27-GFP biosensor (SEQ ID 169-170) in two cell lines (BHK and HeLa) using a library of heavy metal chemical compounds as biological toxin stimulants to stress the cells. Briefly, cells expressing the HSP27-GFP biosensor are plated into 96-well microplates, and allowed to attach. The cells are then treated with a panel of cell stress-inducing compounds. Exclusively cytoplasmic localization of the fusion protein was found in unstimulated cells.

Other similar heat shock protein biosensors (HSP-70, HSC70, and HSF1 fused to GFP) can be used as detectors, and are shown in SEQ ID NO: 171-176.

### *Examples of Classifiers:*

This class of sensors detects the presence of, and further classifies toxins by identifying the cellular pathway(s) perturbed by the toxin. As such, this suite of sensors can detect and/or classify toxins into broad categories, including but not limited to "toxins affecting signal transduction," "toxins affecting the cytoskeleton," and "toxins affecting protein synthesis". Either high throughput or high content screening modes may be used. Classifiers can comprise compounds including but not limited to tubulin, microtubule-associated proteins, actin, actin-binding proteins including but not limited to vinculin,  $\alpha$ -actinin, actin depolymerizing factor/cofilin, profilin, and myosin; NF- $\kappa$ B, I $\kappa$ B, GTP-binding proteins including but not limited to rac, rho, and cdc42, and stress-activated protein kinases including but not limited to p38 mitogen-activated protein kinase.

#### *1. Tubulin-cytoskeleton*

The cell cytoskeleton plays a major role in cellular functions and processes, such as endo- and exocytosis, vesicle transport, and mitosis. Cytoskeleton-affecting

toxins, of proteinaceous and non-proteinaceous form, such as C2 toxin, and several classes of enterotoxins, act either directly on the cytoskeleton, or indirectly via regulatory components controlling the organization of the cytoskeleton. Therefore, measurement of structural changes in the cytoskeleton can provide classification of the toxin into a class of cytoskeleton-affecting toxins. This assay can be conducted in a high content mode, as described previously, or in a high throughput mode. For high throughput as discussed previously.

Such measurements will be valuable for identification of toxins including, but not limited to anti-microtubule agents, agents that generally affect cell cycle progression and cell proliferation, intracellular signal transduction, and metabolic processes.

For microtubule disruption assays, LLC PK cells stably transfected with a tubulin-GFP biosensor plasmid were plated on 96 well cell culture dishes at 50-60% confluence and cultured overnight at 37 °C, 5% CO<sub>2</sub>. A series of concentrations (10-500 nM) of 5 compounds (paclitaxel, curacin A, nocodazole, vinblastine, and colchicine) in normal culture media were freshly prepared from stock, and were added to cell culture dishes to replace the old culture media. The cells were then observed with the cell screening system described above, at a 12 hour time point.

Our data indicate that the tubulin chimera localizes to and assembles into microtubules throughout the cell. The microtubule arrays in cells expressing the chimera respond as follows to a variety of anti-microtubule compounds:

<u>Drug</u>	<u>Response</u>
Vinblastine	Destabilization
Nocodazole	Destabilization
Paclitaxel	Stabilization
Colchicine	Destabilization
Curacin A	Destabilization

Similar data were obtained using cells expressing the tubulin biosensor that were patterned onto cell arrays (such as those described in U.S. Patent Application Serial No. 08/865,341 filed May 29, 1997, incorporated by reference herein in its entirety) and dosed as above.



## 2. NF- $\kappa$ B

NF- $\kappa$ B is cytoplasmic at basal levels of stimulation, but upon insult translocates to the nucleus where it binds specific DNA response elements and activates transcription of a number of genes. Translocation occurs when I $\kappa$ B is degraded by the proteasome in response to specific phosphorylation and ubiquitination events. I $\kappa$ B normally retains NF- $\kappa$ B in the cytoplasm via direct interaction with the protein, and masking of the NLS sequence of NF- $\kappa$ B. Therefore, although not the initial or defining event of the whole signal cascade, NF- $\kappa$ B translocation to the nucleus can serve as an indicator of cell stress.

We have generated an NF- $\kappa$ B-GFP chimera for analysis in live cells. This was accomplished using standard polymerase chain reaction techniques using a characterized NF- $\kappa$ B p65 cDNA purchased from Invitrogen (Carlsbad, CA) fused to an EYFP PCR amplicon that was obtained from Clontech Laboratories (Palo Alto, CA). The resulting chimera is shown in SEQ ID NO:177-178. The two PCR products were ligated into an eukaryotic expression vector designed to produce the chimeric protein at high levels using the ubiquitous CMV promoter.

### NF- $\kappa$ B immunolocalization

For further studies, we characterized endogenous NF- $\kappa$ B activation by immunolocalization in toxin treated cells. The NF- $\kappa$ B antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and secondary antibodies are from Molecular Probes (Eugene, OR).

For the 3T3 and SNB19 cell types, we determined the effective concentrations that yield response levels of 50% of the maximum (EC50), expressed in units of mass per volume (ng/ml) and units of molarity. Based on molecular weights of 17 kD for both TNF $\alpha$  and IL-1 $\alpha$ , the EC50 levels for these two compounds with 3T3 and SNB19 cell types are given in units of molarity in Table 1. Our results demonstrated reproducibility of the relative responses from zero to maximum dose, but from sample to sample there have been occasional shifts in the baseline intensities of the response at zero concentration.

For these experiments, either 10 or 100 TNF $\alpha$ -treated 3T3 or SNB19 cells/well were tested. On the basis of the standard deviations measured for these samples, and by taking t-values for the student's t-test, we have estimated the minimum detectable doses for each case of cell type, compound, number of cells per well, and for different choices of how many wells are sampled per condition. The latter factor determines the number of degrees of freedom that are provided in the sample of data. Increasing the number of wells from 4 to 16, and increasing the number of cells per well from 10 to 100, improves the minimum detectable doses considerably. For 3T3 cells, which show lower minimum detectable doses than the SNB19 cells, and for the case of 1% false negative and 1% false positive rates, we estimate that 100 cells per well and a sampling of 12 or 16 wells are sufficient to detect a dose approximately equal to the EC50 value of 0.15 ng/ml. If the false positive rate is relaxed to 20%, a concentration of approximately half that value can be detected (0.83 ng/ml). One hundred cells can conveniently be sampled over a cell culture surface area of less than 1 mm<sup>2</sup>.

**Table 1.** EC50 levels for TNF $\alpha$  and IL-1 $\alpha$  (based on molecular weights of 17 kD for both)

Compound	Cell Type	EC50 (10 <sup>-12</sup> moles/liter)
TNF $\alpha$	3T3	8.8
	SNB19	5.9
IL-1 $\alpha$	3T3	0.24
	SNB19	59

3. *Phospho-p38 Mitogen Activated Protein Kinase (pp38MAPK)*

MAPKs play a role in not only cell growth and division, but as mediators of cellular stress responses. One MAPK, p38, is activated by chemical stress inducers such as hyper-osmolar sorbitol, hydrogen peroxide, arsenite, cadmium ions, anisomycin, sodium salicylate, and LPS. Activation of p38 is also accompanied by its translocation into the nucleus from the cytoplasm.

MAPK p38 lies in a pathway that is a cascade of kinases. Thus, p38 is a substrate of one or more kinases, and it acts to phosphorylate one or more substrates in time and space within the living cell.

The assay we present here measures, as one of its parameters, p38 activation using immunolocalization of the phosphorylated form of p38 in toxin-treated cells. The assay was developed to be flexible enough to include the simultaneous measurement of other parameters within the same individual cells. Because the signal transduction pathway mediated by the transcription factor NF- $\kappa$ B is also known to be involved in the cell stress response, we included the activation of NF- $\kappa$ B as a second parameter in the same assay.

Our experiments demonstrate an immunofluorescence approach can be used to measure p38 MAPK activation either alone or in combination with NF- $\kappa$ B activation in the same cells. Multiple cell types, model toxins, and antibodies were tested, and significant stimulation of both pathways was measured in a high-content mode. The phospho-p38 antibodies used in this study were purchased from Sigma Chemical Company (St. Louis, MO). We report that at least two cell stress signaling pathways can not only be measured simultaneously, but are differentially responsive to classes of model toxins. Figure 36 shows the differential response of the p38 MAPK and NF- $\kappa$ B pathways across three model toxins and two different cell types. Note that when added alone, three of the model toxins (IL1 $\alpha$ , TNF $\alpha$  and Anisomycin) can be differentiated by the two assays as activators of specific pathways.

#### I $\kappa$ B chimera

I $\kappa$ B degradation is the key event leading to nuclear translocation of NF- $\kappa$ B and activation of the NF $\kappa$ B-mediated stress response. We have chosen this sensor to complement the NF- $\kappa$ B sensor as a *classifier* in a high-throughput mode: the measurement of loss of signal due to degradation of the I $\kappa$ B-GFP fusion protein requires no spatial resolution within individual cells, and as such we envision I $\kappa$ B degradation measurements being made rapidly on an entire cell substrate.

This biosensor is based on fusion of the first 60 amino acids of I $\kappa$ B to the Fred25 variant of GFP. SEQ ID 179-180 This region of I $\kappa$ B contains all the regulatory

sequences, including phosphorylation sites and ubiquitination sites, necessary to confer proteasome degradation upon the biosensor. Knowing this, stimulation of any pathway that would typically lead to NFkB translocation results in degradation of this biosensor. Monitoring the fluorescence intensity of cells expressing Ikb-GFP identifies the degradation process.

### Examples of Identifiers:

In our toxin identification strategy, the first two levels of characterization ensure a rapid readout of toxin class without sacrificing the ability to detect many new mutant toxins or dissect several complex mixtures of known toxins. The third level of biosensors are identifiers, which can identify a specific toxin or group of toxins. In one embodiment, an identifier comprises a protease biosensor that responds to the activity of a specific toxin. Other identifiers are produced with reporters/biosensors specific to their activities. These include, but are not limited to post-translational modifications such as phosphorylation or ADP-ribosylation, translocation between cellular organelles or compartments, effects on specific organelles or cellular components (for example, membrane permeabilization, cytoskeleton rearrangement, etc.)

ADP-ribosylating toxins – These toxins include Pseudomonas toxin A, diphtheria toxin, botulinum toxin, pertussis toxin, and cholera toxin. For example, C. botulinum C2 toxin induces the ADP-ribosylation of Arg177 in the cytoskeletal protein actin, thus altering its assembly properties. Besides the construction of a classifier assay to measure actin-cytoskeleton regulation, an identifier assay can be constructed to detect the specific actin ADP-ribosylation. Because the ADP-ribosylation induces a conformational change that no longer permits the modified actin to polymerize, this conformational change can be detected intracellularly in several possible ways using luminescent reagents. For example, actin can be luminescently labeled using a fluorescent reagent with an appropriate excited state lifetime that allows for the measurement of the rotational diffusion of the intracellular actin using steady state fluorescence anisotropy. That is, toxin-modified actin will no longer be able to assemble into rigid filaments and will therefore produce only luminescent signals with

relatively low anisotropy, which can be readily measured with an imaging system. In another embodiment, actin can be labeled with a polarity-sensitive fluorescent reagent that reports changes in actin-conformation through spectral shifts of the attached reagent. That is, toxin-treatment will induce a conformational change in intracellular actin such that a ratio of two fluorescence wavelengths will provide a measure of actin ADP-ribosylation.

Cytotoxic phospholipases – Several gram-positive bacterial species produce cytotoxic phospholipases. For example, *Clostridium perfringens* produces a phospholipase C specific for the cleavage of phosphoinositides. These phosphoinositides (e.g., inositol 1,4,5-trisphosphate) induce the release of calcium ions from intracellular organelles. An assay that can be conducted as either high-content or high-throughput can be constructed to measure the release of calcium ions using fluorescent reagents that have altered spectral properties when complexed with the metal ion. Therefore, a direct consequence of the action of a phospholipase C based toxin can be measured as a change in cellular calcium ion concentration.

Exfoliative toxins – These toxins are produced by several *Staphylococcal* species and can consist of several serotypes. A specific identifier for these toxins can be constructed by measuring the morphological changes in their target organelle, the desmosome, which occur at the junctions between cells. The exfoliative toxins are known to change the morphology of the desmosomes into two smaller components called hemidesmosomes. In the high-content assay for exfoliative toxins, epithelial cells whose desmosomes are luminescently labeled are subjected to image analysis. A method that detects the morphological change between desmosomes and hemidesmosomes is used to quantify the activity of the toxins on the cells.

Most of these identifiers can be used in high throughput assays requiring no spatial resolution, as well as in high content assays.

Several biological threat agents act as specific proteases, and thus we have focused on the development of fluorescent protein biosensors that report the proteolytic cleavage of specific amino acid sequences found within the target proteins.

A number of such protease biosensors (including FRET biosensors) are disclosed above, such as the caspase biosensors, anthrax, tetanus, Botulinum, and the

zinc metalloproteases. FRET is a powerful technique in that small changes in protein conformation, many of which are associated with toxin activity, can not only be measured with high precision in time and space within living cells, but can be measured in a high-throughput mode, as discussed above.

5 As described above, one of skill in the art will recognize that the protease biosensors of this aspect of the invention can be adapted to report the activity of any protease, by a substitution of the appropriate protease recognition site in any of the constructs (see **Figure 29B**). As disclosed above, these biosensors can be used in high-content or high throughput screens to detect *in vivo* activation of enzymatic activity by  
10 toxins, and to identify specific activity based on cleavage of a known recognition motif. These biosensors can be used in both live cell and fixed end-point assays, and can be combined with additional measurements to provide a multi-parameter assay.

#### **Anthrax LF**

15 Anthrax is a well-known agent of biological warfare and is an excellent target for development of a biosensor in the *identifier* class. Lethal factor (LF) is one of the protein components that confer toxicity to anthrax, and recently two of its targets within cells were identified. LF is a metalloprotease that specifically cleaves Mek1 and Mek2 proteins, kinases that are part of the MAP-kinase signaling pathway. Construction of  
20 lethal factor protease biosensors are described above. (SEQ ID NO:7-8; 9-10) Green fluorescent protein (GFP) is fused in-frame at the amino terminus of either Mek1 or Mek2 (or both), resulting in a chimeric protein that is retained in the cytoplasm due to the presence of a nuclear export sequence (NES) present in both of the target molecules. Upon cleavage by active lethal factor, GFP is released from the chimera and  
25 is free to diffuse into the nucleus. Therefore, measuring the accumulation of GFP in the nucleus provides a direct measure of LF activity on its natural target, the living cell.

While a preferred form of the invention has been shown in the drawings and described, since variations in the preferred form will be apparent to those skilled in the art, the invention should not be construed as limited to the specific form shown and  
30 described, but instead is as set forth in the claims.

### CLAIMS

We claim:

1. An automated method for cell based toxin characterization comprising
  - providing an array of locations containing cells to be treated with a test  
5 substance, wherein the cells possess at least a first luminescent reporter molecule comprising a detector and a second luminescent reporter molecule selected from the group consisting of a classifier or an identifier;
  - contacting the cells with the test substance either before or after possession of the first and second luminescent reporter molecules by the cells; wherein the  
10 localization, distribution, structure, or activity of the first and second luminescent reporter molecule is modified when the cell is contacted with the toxin,
  - imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector;
  - converting the luminescent signals from the detector into digital data;
  - 15 -utilizing the digital data from the detector to automatically measure the localization, distribution, or activity of the detector on or in the cell, wherein a change in the localization, distribution, structure or activity of the detector indicates the presence of a toxin in the test substance;
  - selectively imaging or scanning the locations containing cells that were  
20 contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the second reporter molecule;
  - converting the luminescent signals from the second luminescent reporter molecule into digital data;
  - utilizing the digital data from the second luminescent reporter molecule to  
25 automatically measure the localization, distribution, or activity of the classifier or identifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the classifier identifies a cell pathway that is perturbed by the toxin present in the test substance, or wherein a change in the localization, distribution, structure or activity of the identifier identifies the specific toxin or group of toxins that  
30 are present in the test substance.

2. The method of claim 1 wherein the second luminescent reporter molecule is a classifier, and the digital data derived from the classifier is used to select an appropriate identifier for identification of the specific toxin or group of toxins.

5 3. An automated method for cell based toxin characterization comprising

-providing an array of locations containing cells to be treated with a test substance, wherein the cells possess at least a first luminescent reporter molecule comprising a detector, a second luminescent reporter molecule comprising a classifier, and a third luminescent reporter molecule comprising an identifier;

10 -contacting the cells with the test substance either before or after possession of the first second, and third luminescent reporter molecules by the cells; wherein the localization, distribution, structure, or activity of the first, second, and third luminescent reporter molecules is modified when the cell is contacted with the toxin,

-imaging or scanning multiple cells in each of the locations containing multiple  
15 cells to obtain luminescent signals from the detector;

-converting the luminescent signals from the detector into digital data;

-utilizing the digital data from the detector to automatically measure the localization, distribution, or activity of the detector on or in the cell, wherein a change in the localization, distribution, structure or activity of the detector indicates the  
20 presence of a toxin in the test substance;

-selectively imaging or scanning the locations containing cells that were contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the classifier;

-converting the luminescent signals from the classifier into digital data;

25 -utilizing the digital data from the classifier to automatically measure the localization, distribution, or activity of the classifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the classifier identifies a cell pathway that is perturbed by the toxin present in the test substance;

30 -selectively imaging or scanning the locations containing cells that were contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the identifier;

-converting the luminescent signals from the identifier into digital data; and



-utilizing the digital data from the identifier to automatically measure the localization, distribution, or activity of the identifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the identifier identifies the specific toxin or group of toxins that is present in the test substance.

5

4. The method of claim 3 wherein the digital data derived from the classifier is used to select an appropriate identifier for identification of the specific toxin or group of toxins.

10

5. The method of any one of claim 1-4 wherein the detector comprises a molecule selected from the group consisting of heat shock proteins and compounds that respond to changes in mitochondrial membrane potential, intracellular free ion concentration, cytoskeletal organization, general metabolic status, cell cycle timing events, and organellar structure and function.

15

6. The method of any one of claim 1-5 wherein the classifier comprises a molecule selected from the group consisting of tubulin, microtubule-associated proteins, actin, actin-binding proteins, NF- $\kappa$ B, I $\kappa$ B, and stress-activated kinases.

20

7. The method of any one of claim 1-6 wherein the cell pathway is selected from the group consisting of cell stress pathways, cell metabolic pathways, cell signaling pathways, cell growth pathways, and cell division pathways.

25

8. The method of claim 1, wherein the second luminescent reporter molecule is an identifier, and the identifier identifies a toxin or group of toxins selected from the group consisting of proteases, ADP-ribosylating toxins, cytotoxic phospholipases, and exfoliative toxins.

30

9. The method of any one of claim 3-7, wherein the identifier identifies a toxin or group of toxins selected from the group consisting of proteases, ADP-ribosylating toxins, cytotoxic phospholipases, and exfoliative toxins.

10. The method of any of claims 1-9 wherein the change in the localization, distribution, structure or activity of the first, second, or third luminescent reporter molecules is selected from the group consisting of cytoplasm to nucleus translocation, nucleus or nucleolus to cytoplasm translocation, receptor internalization, mitochondrial  
5 membrane potential, loss of signal, the spectral response of the reporter molecule, phosphorylation, intracellular free ion concentration, cell size, cell shape, cytoskeleton organization, metabolic processes, cell motility, cell substrate attachment, cell cycle events, and organellar structure and function.

10 11. The method of any one of claims 1-10, wherein the imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector is carried out in a high throughput mode.

12. The method of any one of claims 1-10, wherein the imaging or scanning  
15 multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector is carried out in a high content mode.

13. The method of claim 1-10 wherein the selective imaging or scanning of the locations containing cells that were contacted with test sample indicated to have a toxin  
20 in it to obtain luminescent signals from the second or third reporter molecule is carried out in a high throughput mode.

14. The method of claim 1-10 wherein the selective imaging or scanning of the locations containing cells that were contacted with test sample indicated to have a toxin  
25 in it to obtain luminescent signals from the second or third reporter molecule is carried out in a high content mode.

15. The method of any one of claims 1-14 further comprising providing a digital storage media for data storage and archiving.

16. The method of claim 15 further comprising a means for automated control, acquisition, processing and display of results.

17. A computer readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the method of any one of claims 1-16, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a means for moving the stage or the optical system, a digital camera, a means for directing light emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera.

18. A kit for cell based toxin detection comprising:

(a) at least one reporter molecule, wherein the localization, distribution, structure, or activity of the reporter molecule is modified when the cell is contacted with a toxin;

(b) instructions for using the reporter molecule to carry out the method of any one of claims 1-16 to detect toxins in a test substance.

19. The kit of claim 18 further comprising the computer readable storage medium of claim 17.

20. An automated method for cell based toxin characterization comprising

-providing a first array of locations containing cells to be treated with a test substance, wherein the cells possess a least a first luminescent reporter molecule comprising a reporter molecule selected from the group consisting of detectors and classifiers;

-contacting the cells with the test substance either before or after possession of the first luminescent reporter molecule by the cells; wherein the localization, distribution, structure, or activity of the first luminescent reporter molecule is modified when the cell is contacted with the toxin,

-imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector;

-converting the luminescent signals from the detector into digital data;

-utilizing the digital data from the detector to automatically measure the localization, distribution, or activity of the detector on or in the cell, wherein a change in the localization, distribution, structure or activity of the detector indicates the presence of a toxin in the test substance,

5        -providing a second array of locations containing cells to be treated with the test substance, wherein the cells possess a least a second luminescent reporter molecule comprising a reporter molecule selected from the group consisting of classifiers and identifiers, and wherein the second array of locations containing cells can comprise either the same or a different cell type as the first array of locations containing cells;

10       -contacting the second array of locations containing cells with the test substance either before or after possession of the second luminescent reporter molecule by the cells; wherein the localization, distribution, structure, or activity of the second luminescent reporter molecule is modified when the cell is contacted with the toxin;

15       -utilizing the digital data from the second luminescent reporter molecule to automatically measure the localization, distribution, or activity of the classifier or identifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the classifier identifies a cell pathway that is perturbed by the toxin present in the test substance, or wherein a change in the localization, distribution, structure or activity of the identifier identifies the specific toxin or group of toxins that  
20       are present in the test substance.

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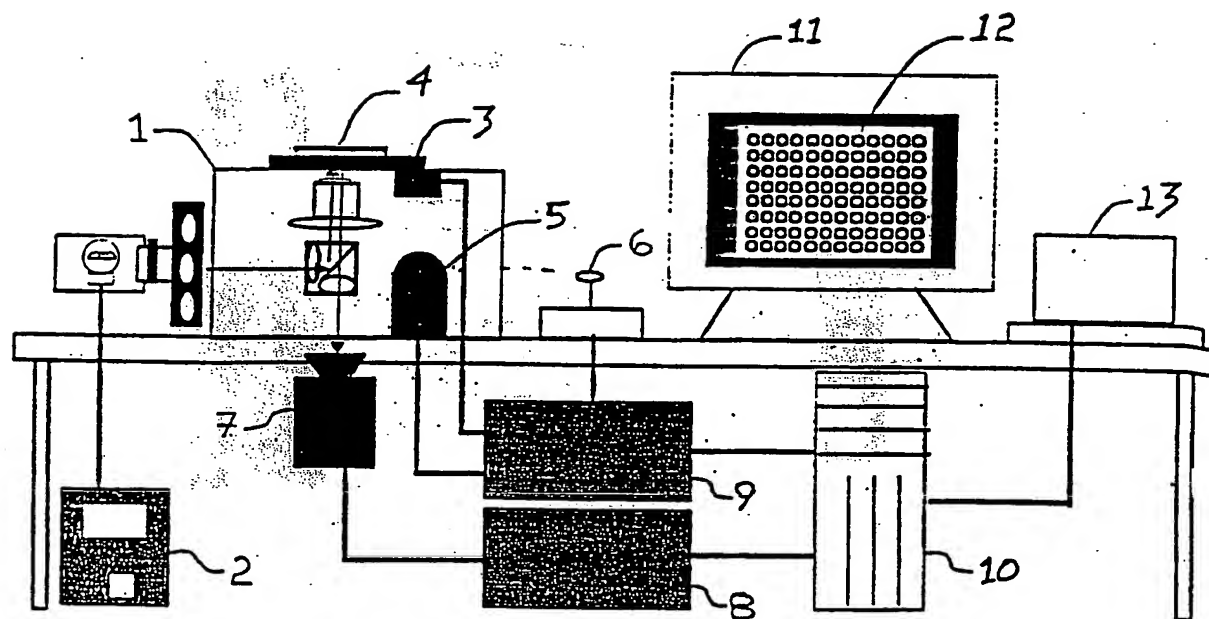


FIGURE 1

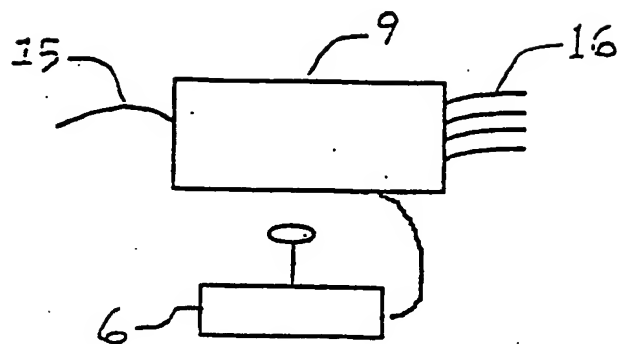
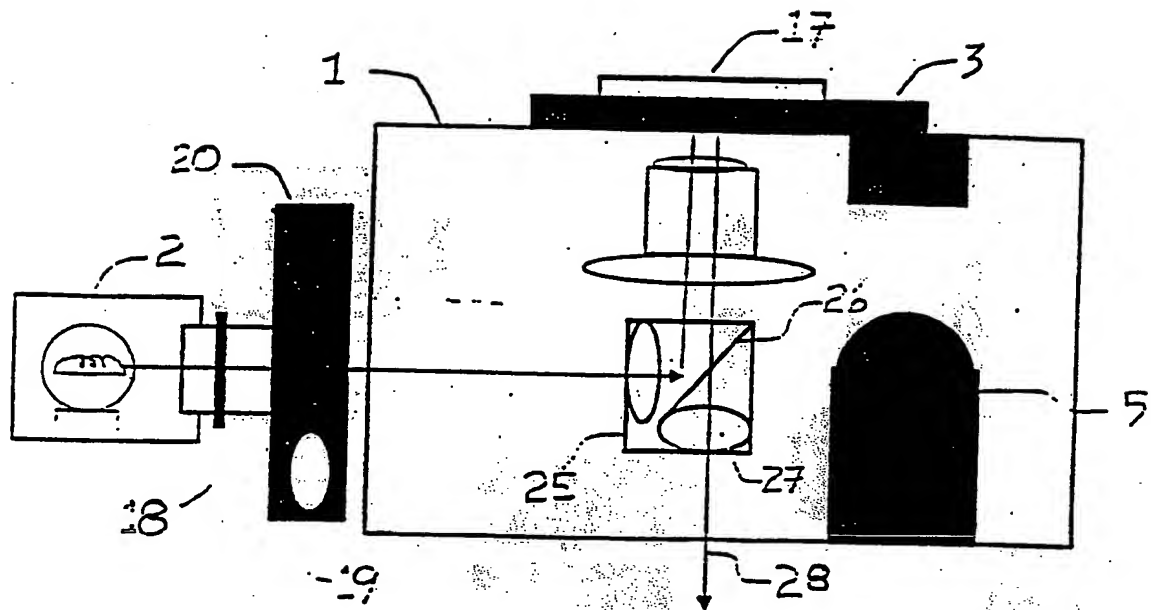


FIGURE 2

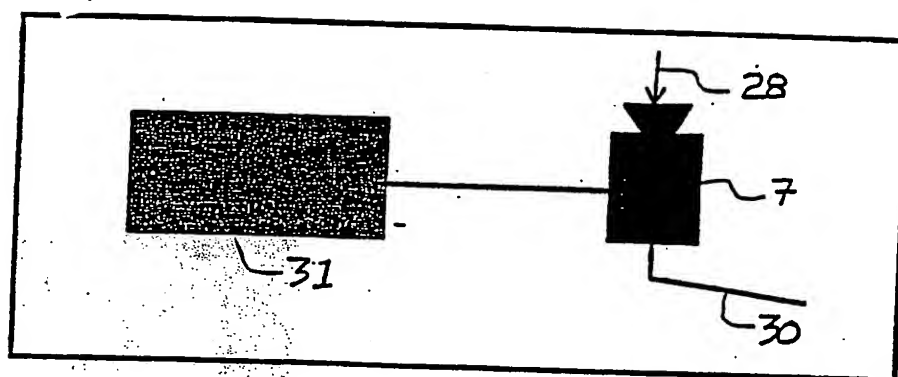


FIGURE 3

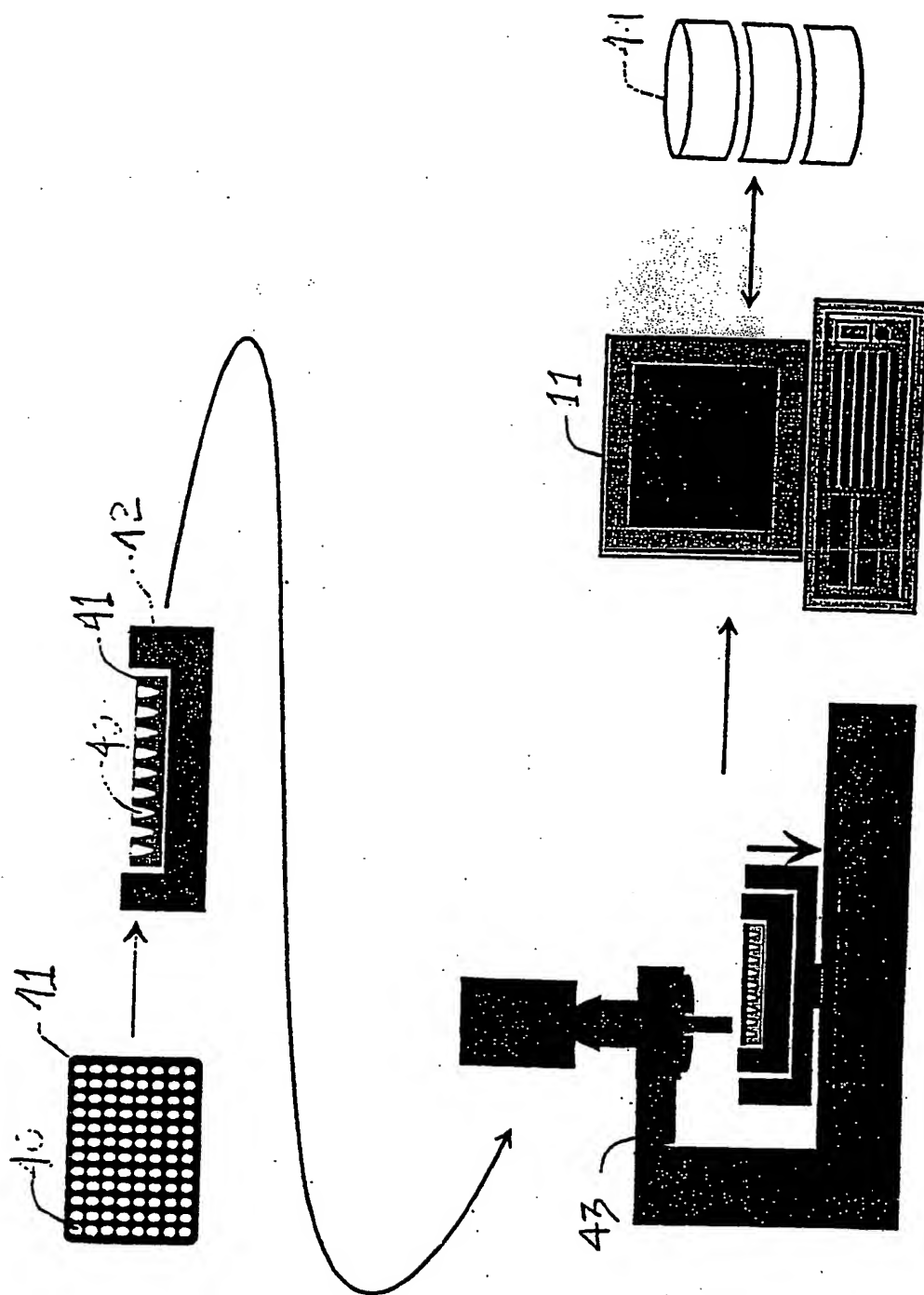
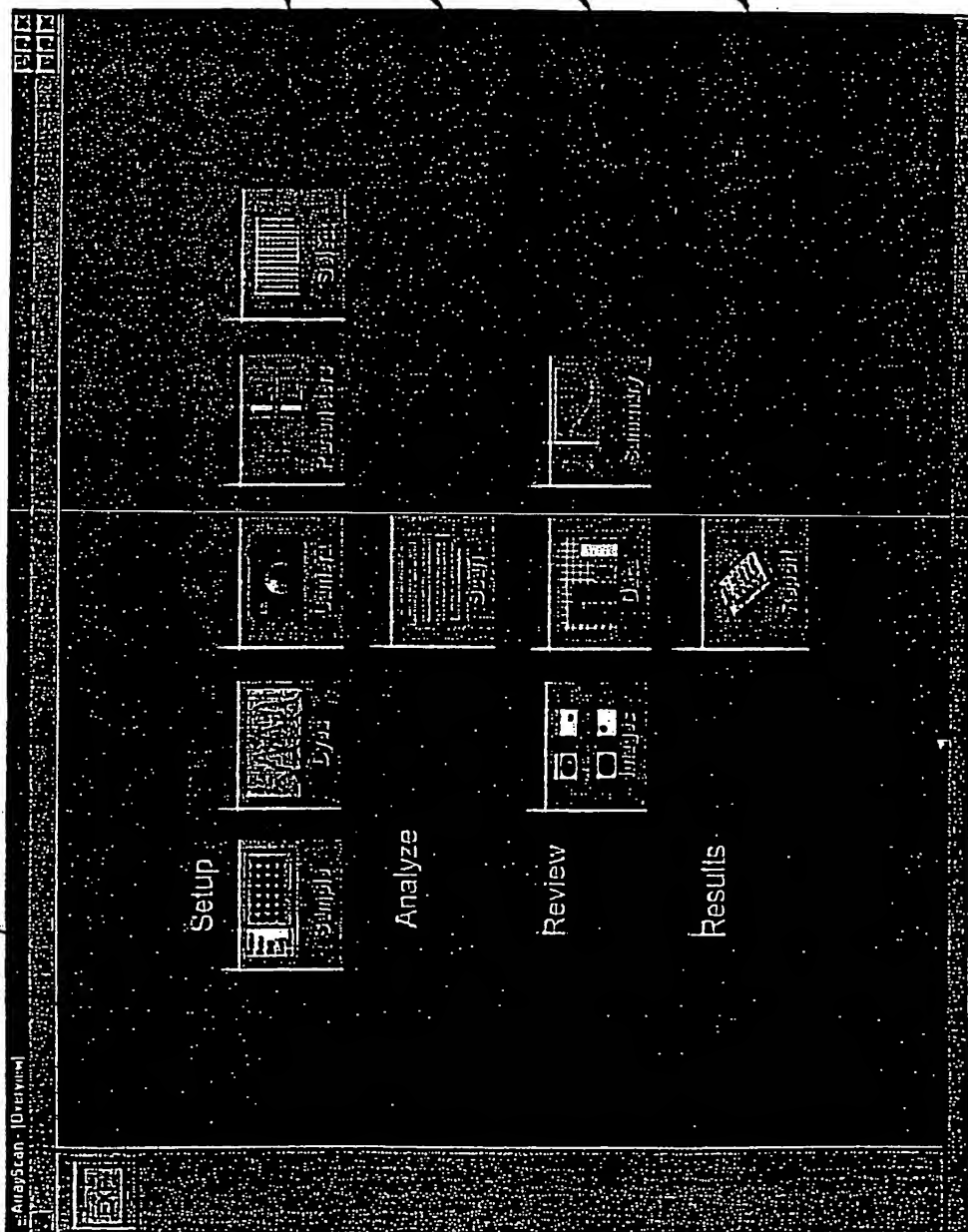


FIGURE 4





**FIGURE 5**

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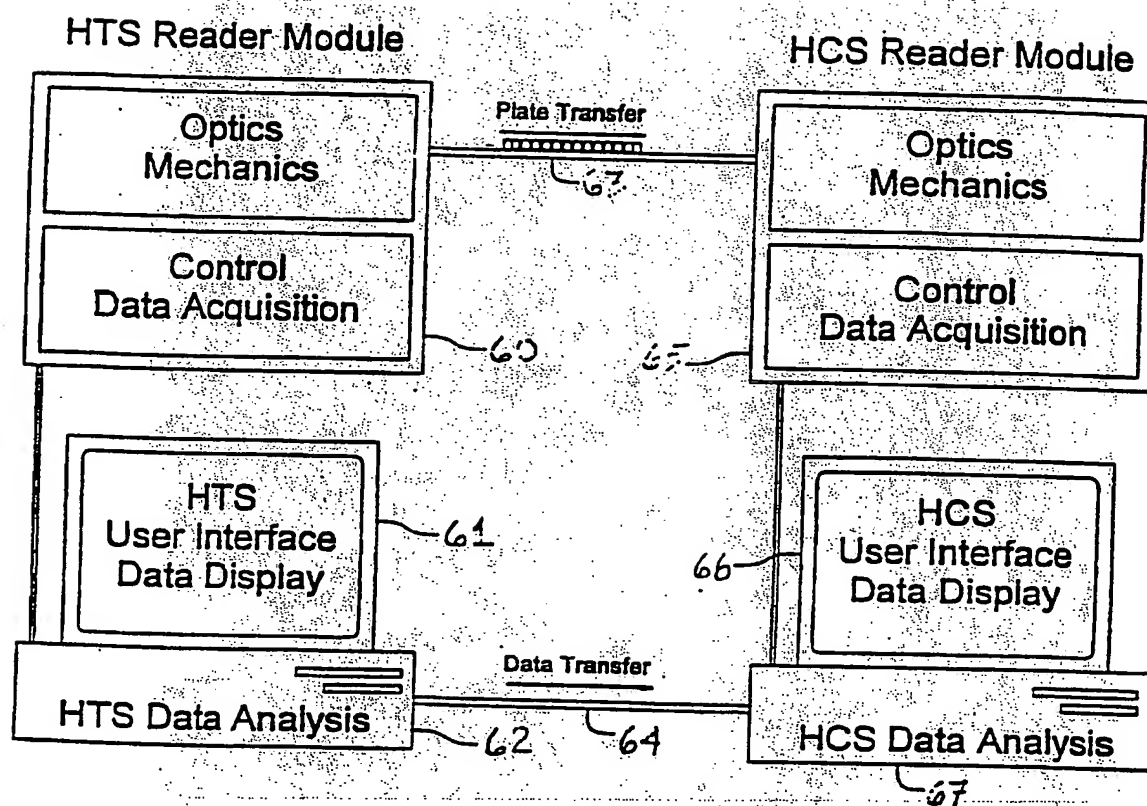


FIG. 6

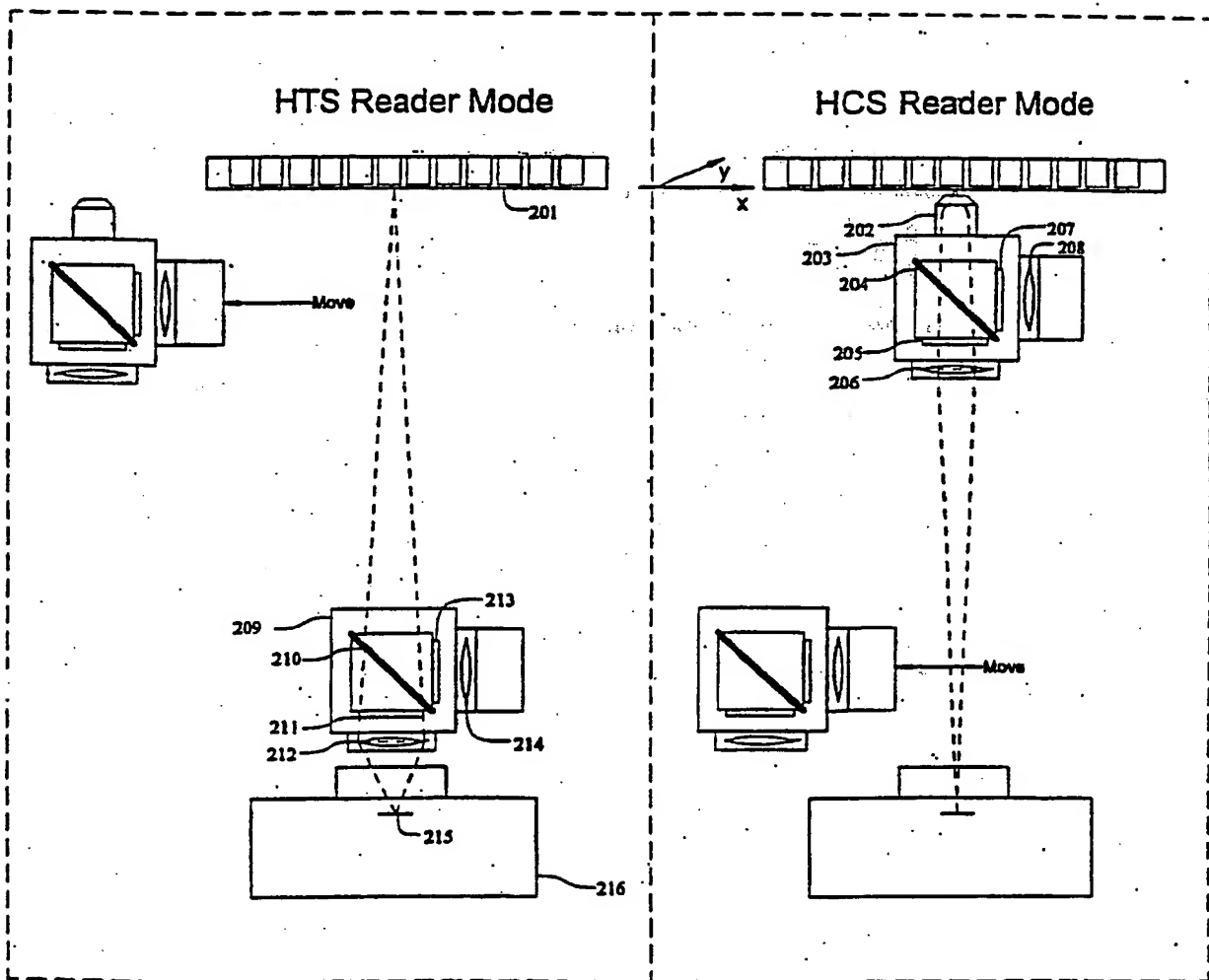
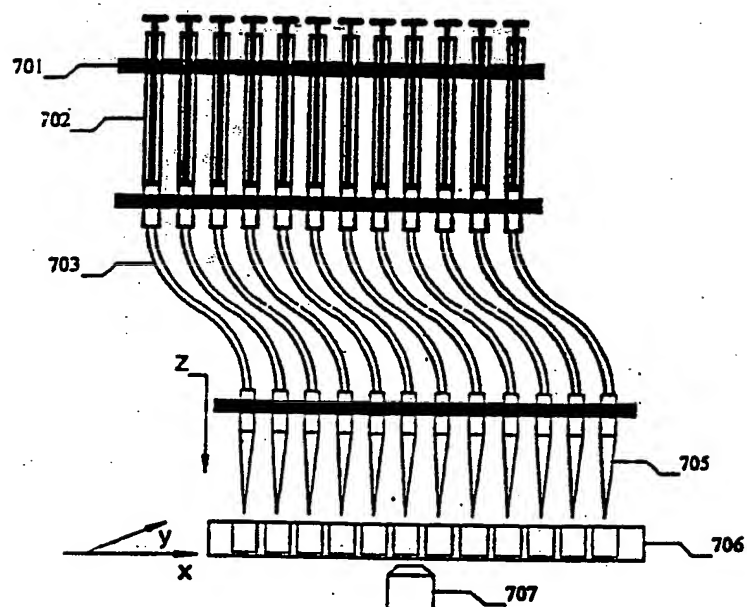


FIGURE 7

## Fluid Delivery System for Cell Based Screening System

**FIGURE 8**

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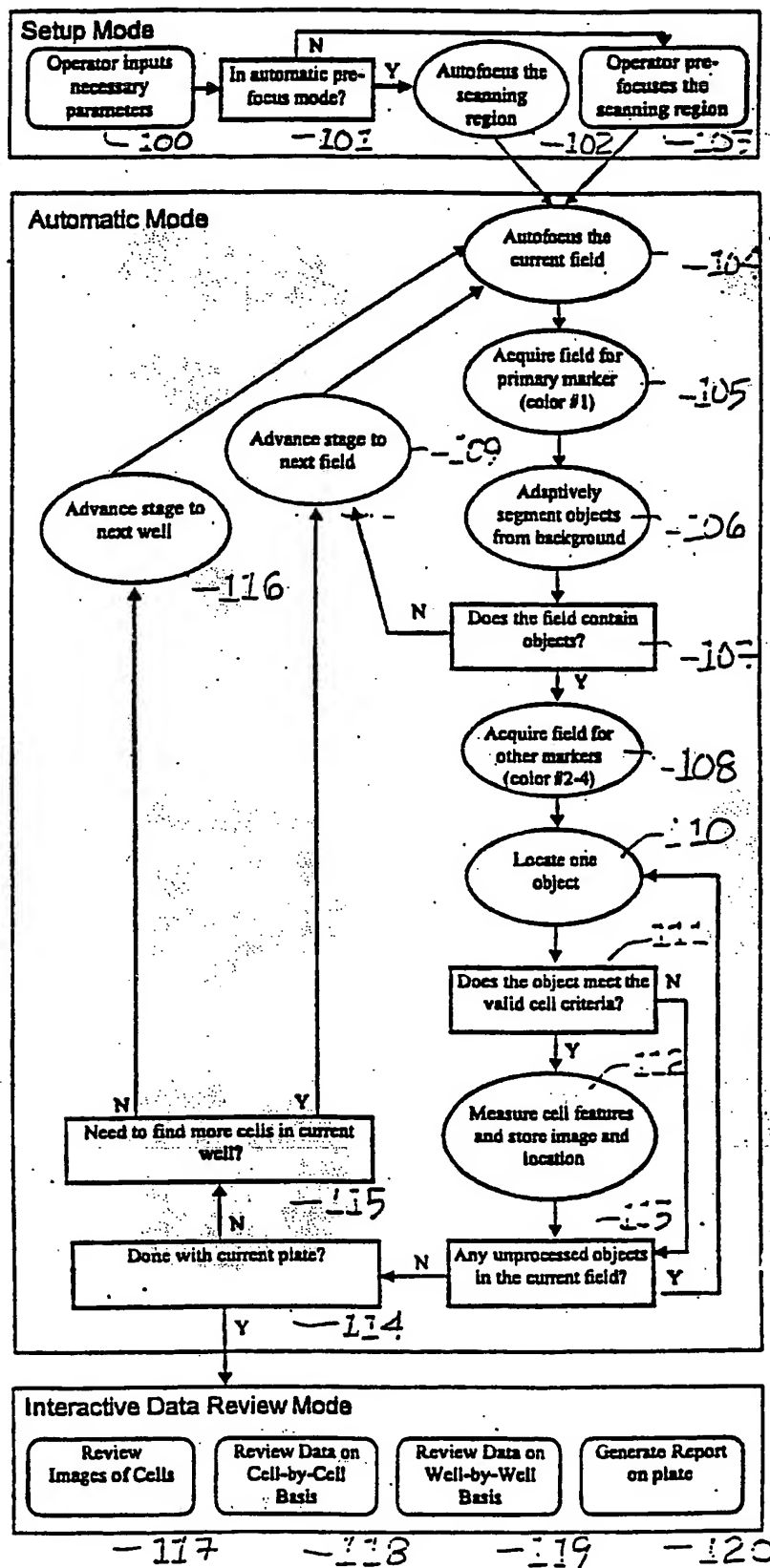


FIGURE 1

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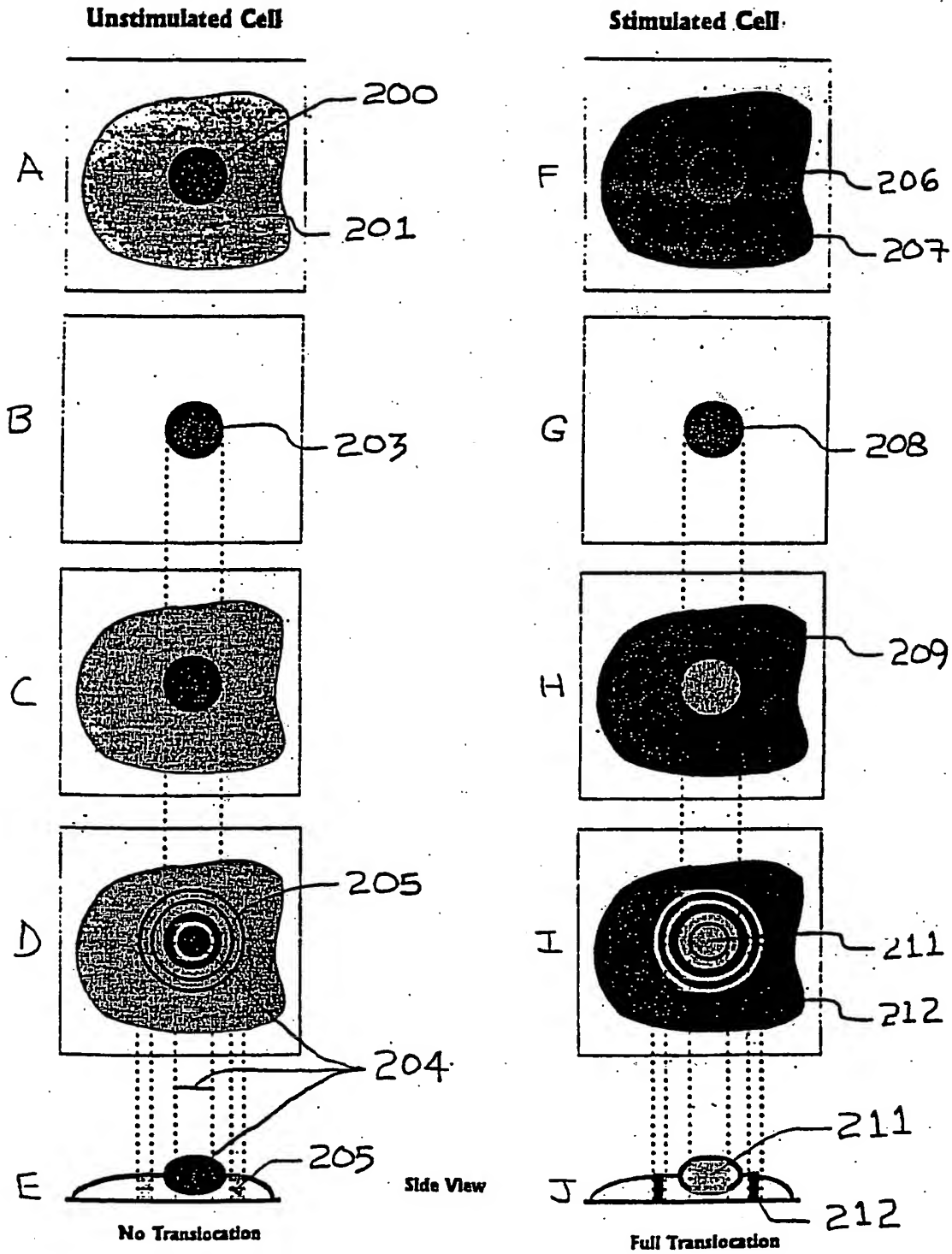
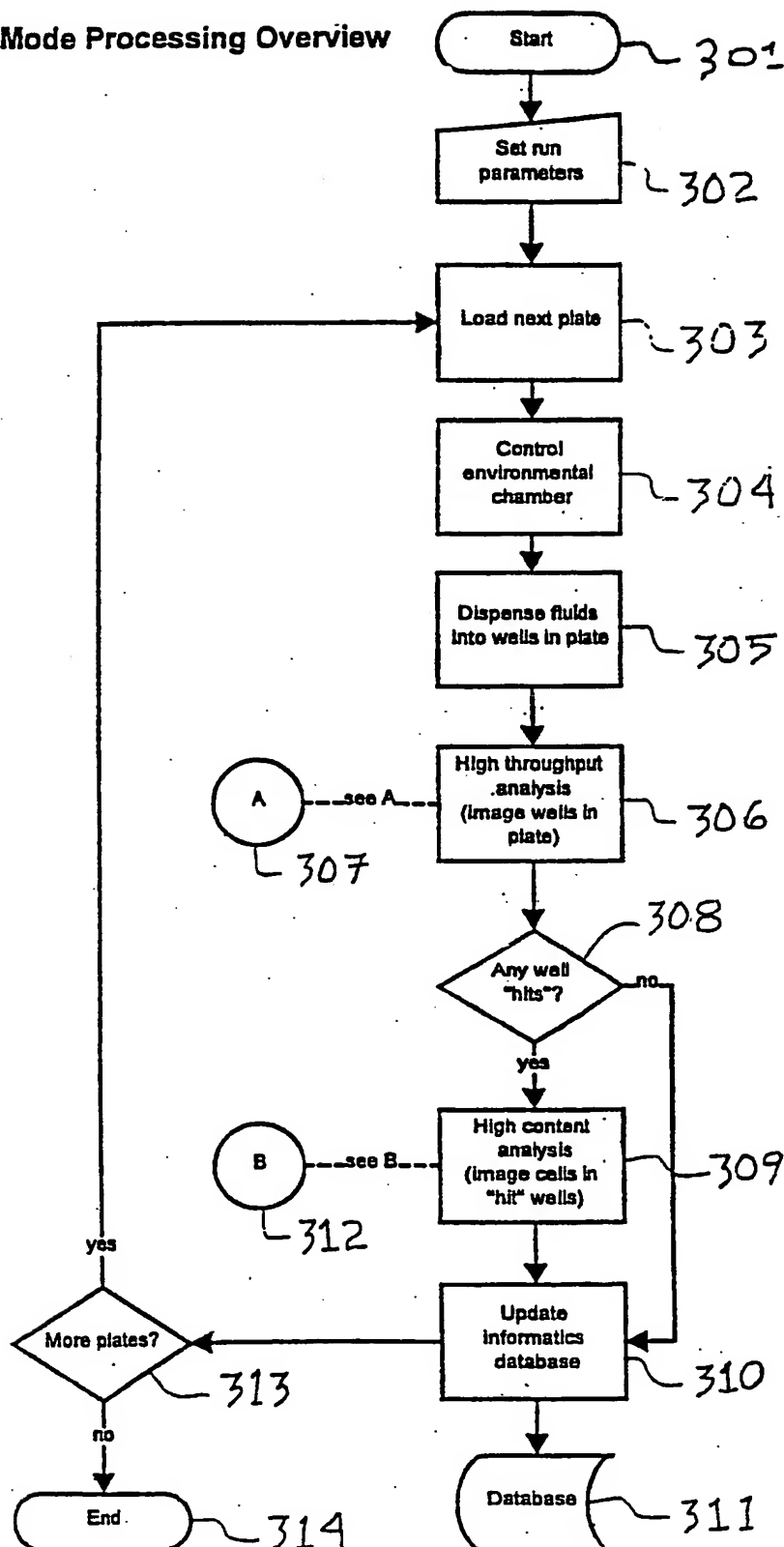


FIGURE 10

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## Dual Mode Processing Overview



Symbol key:

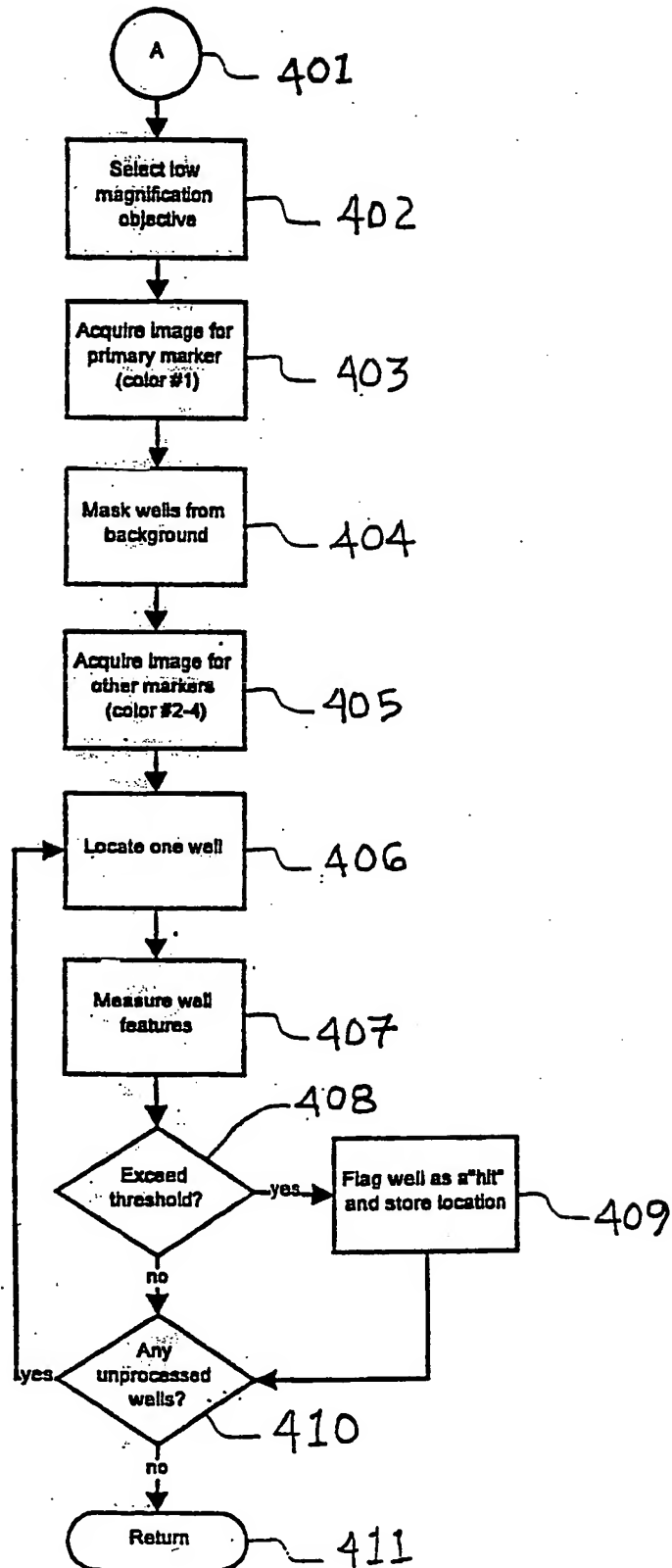
Manual input

Automatic Process

Decision

Connector

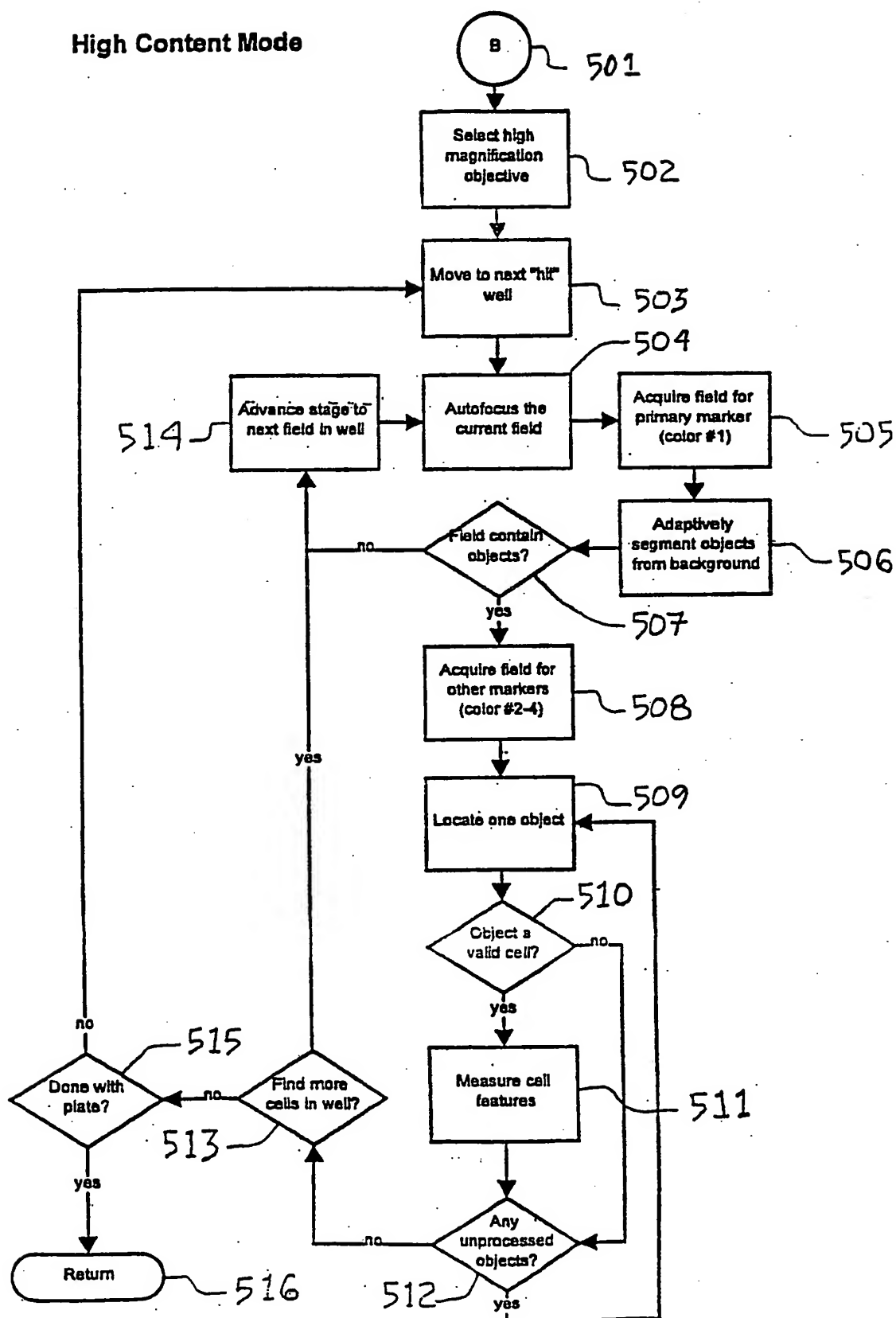
12/50

**High Throughput Mode**



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## High Content Mode



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## Kinetic Analysis Mode

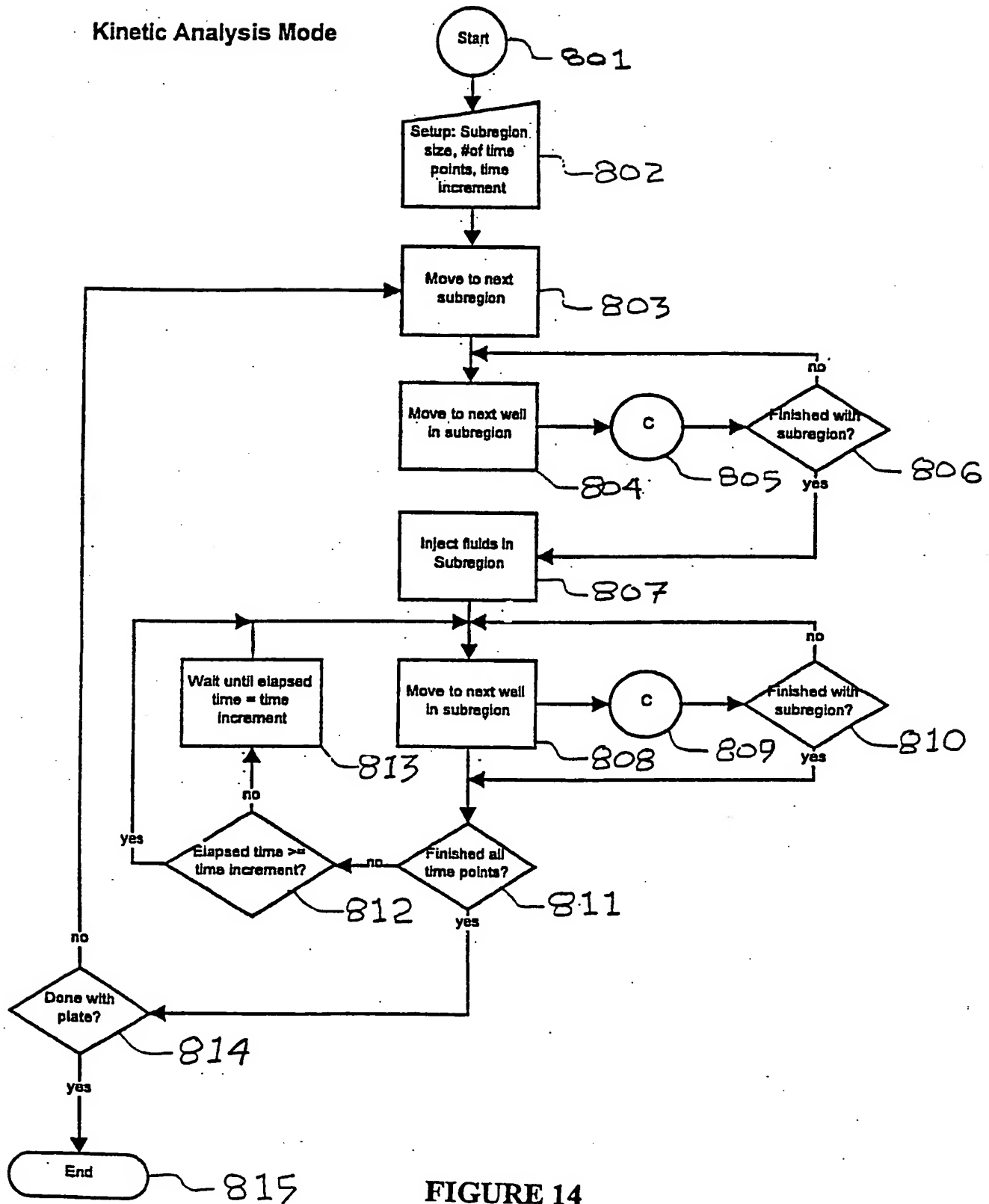


FIGURE 14

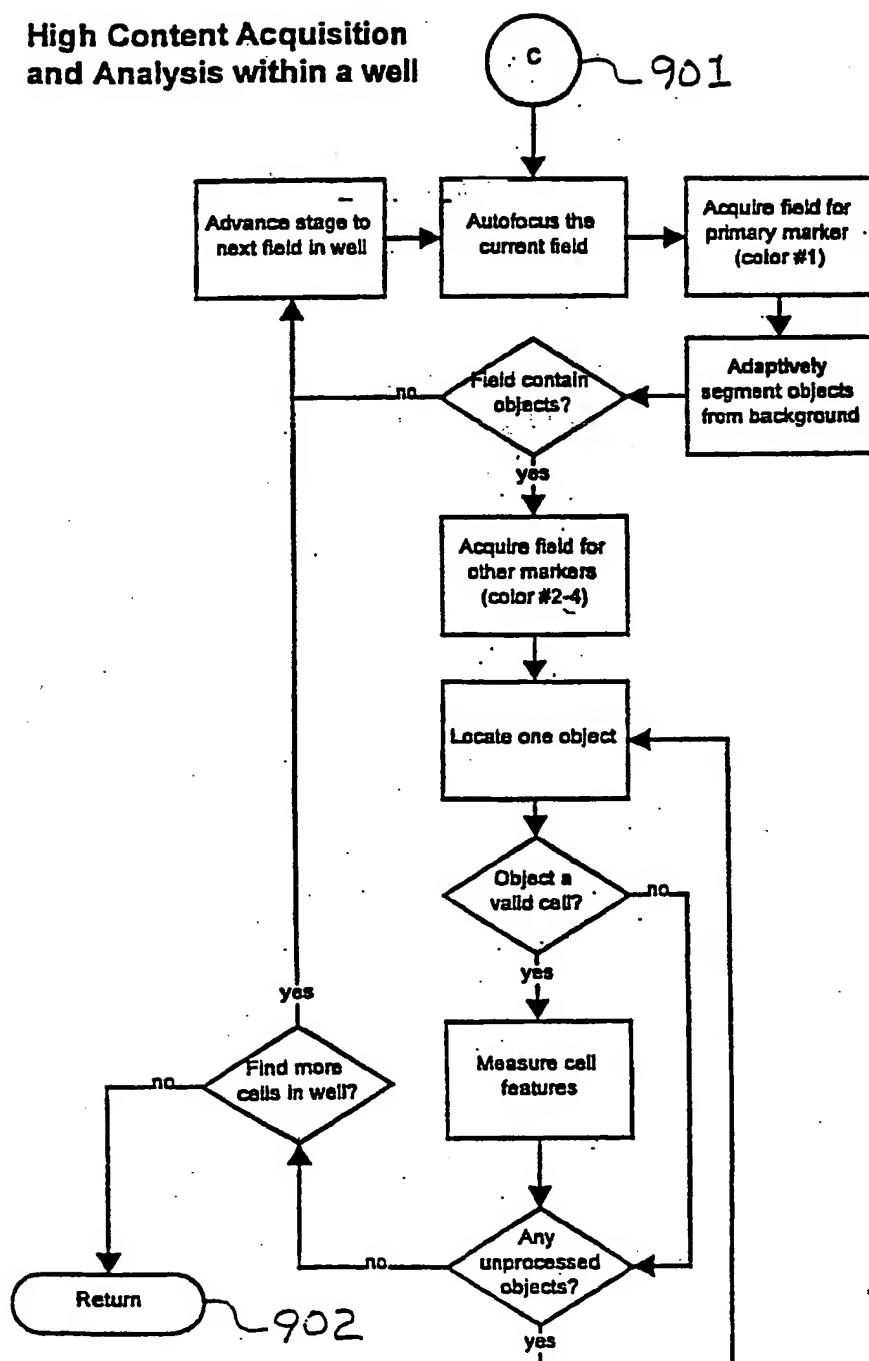
**High Content Acquisition  
and Analysis within a well**

Fig 15

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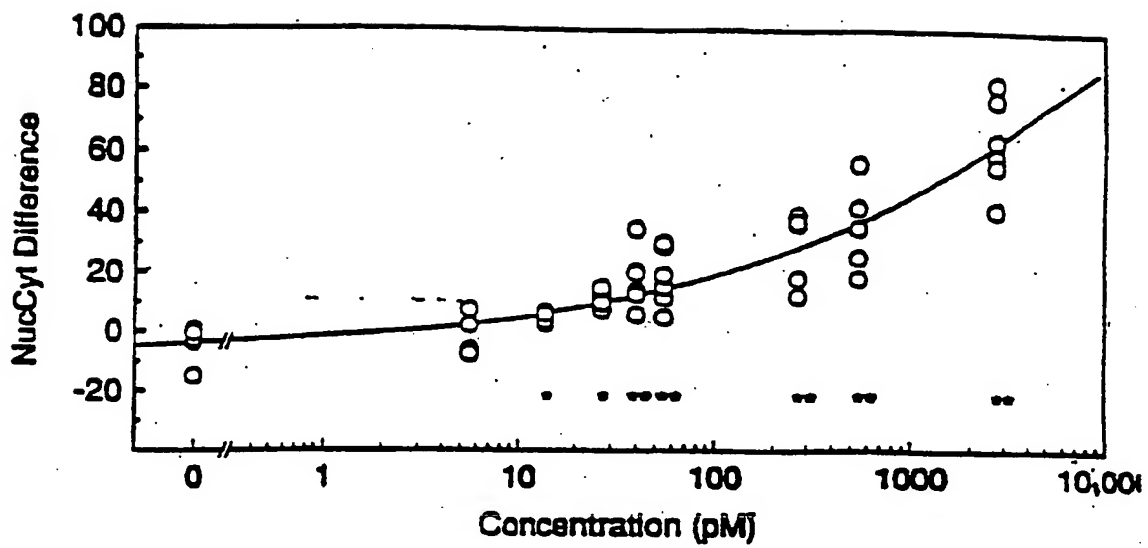


FIGURE 16

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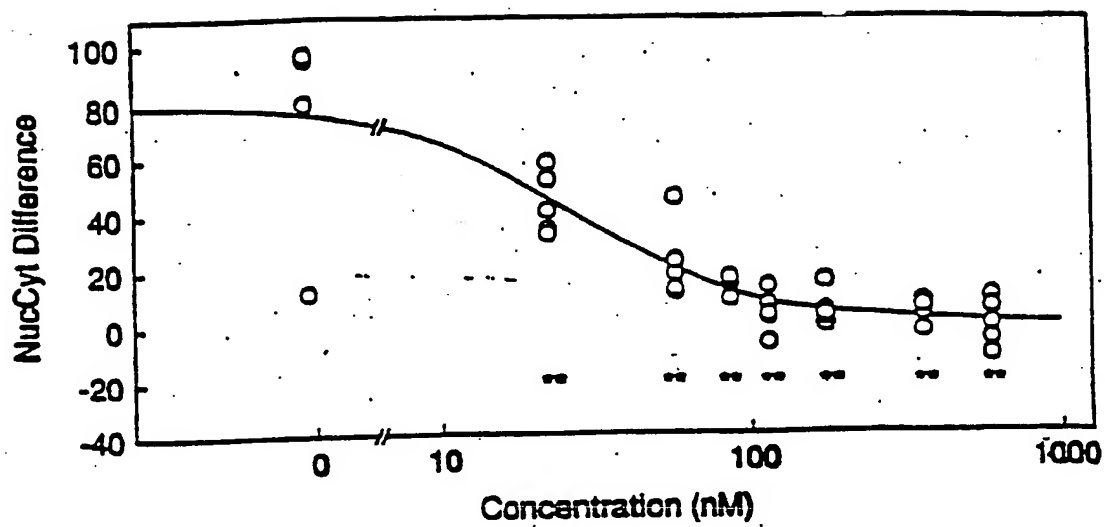
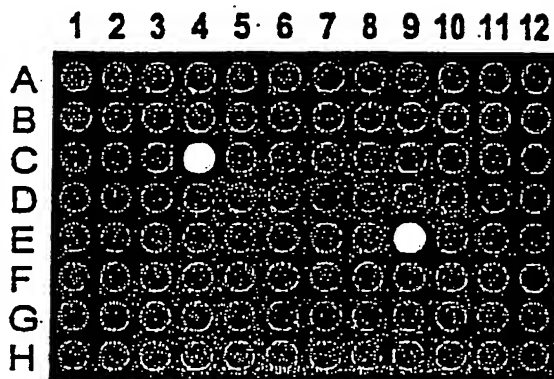


FIGURE 17



**Low Resolution  
Well Data**



Intensity  
Analysis

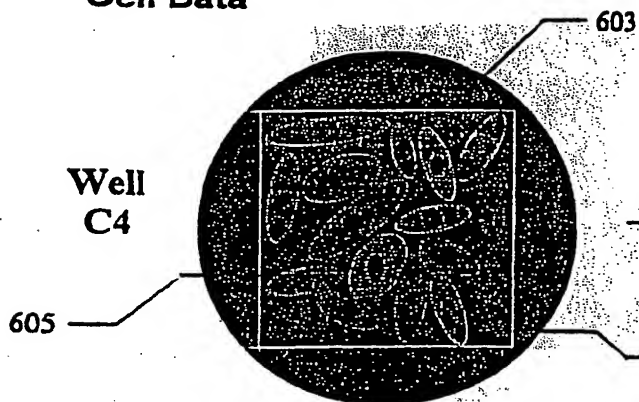
Array of  
"Hit" Wells

[C4, E9]

602

601

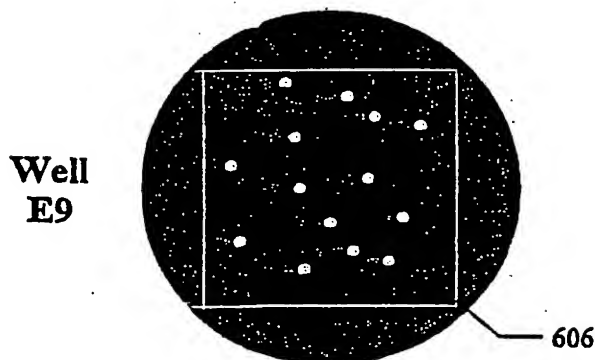
**High Resolution  
Cell Data**



Spatial  
Analysis

No Translocation

604



Spatial  
Analysis

Translocation

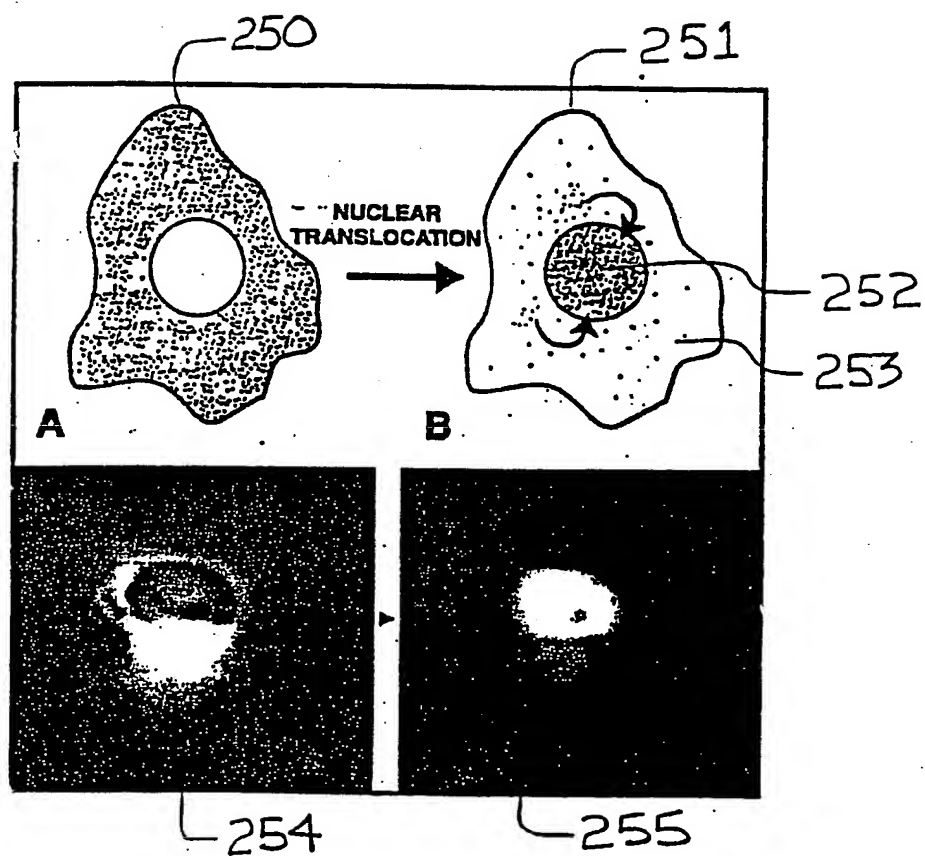


FIGURE 20



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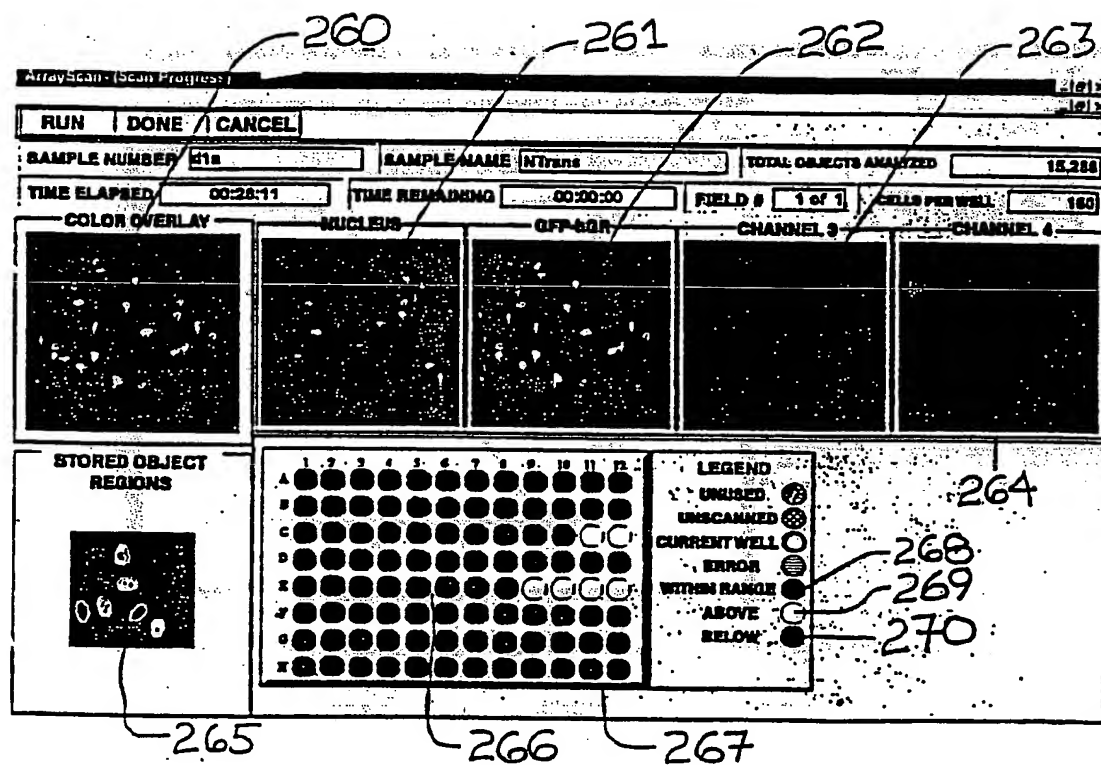
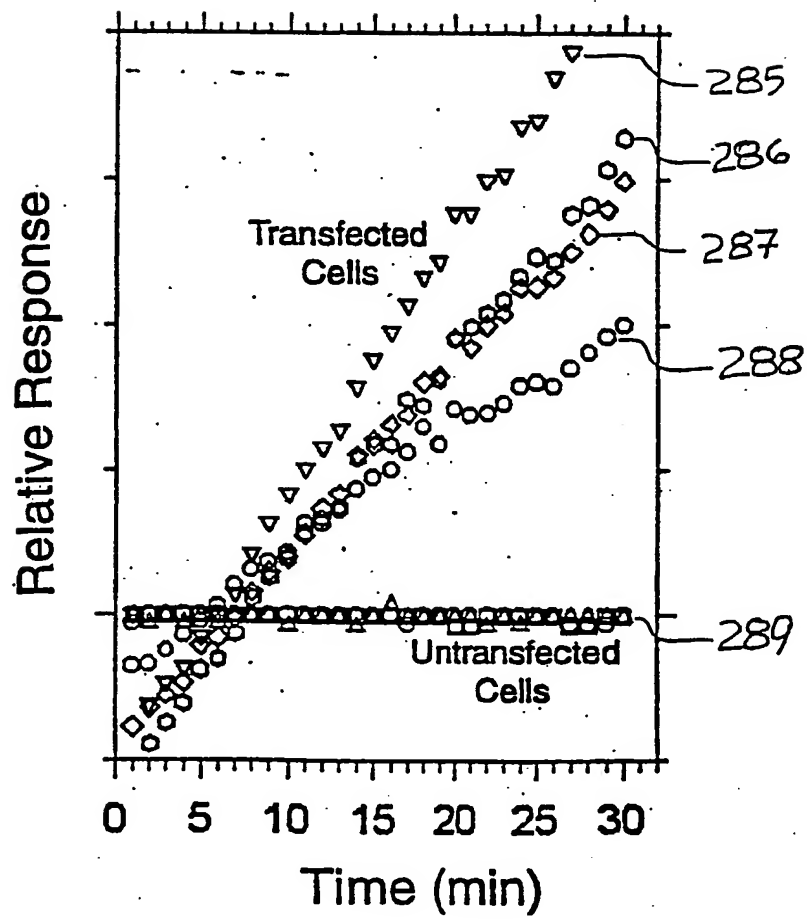


FIGURE 21



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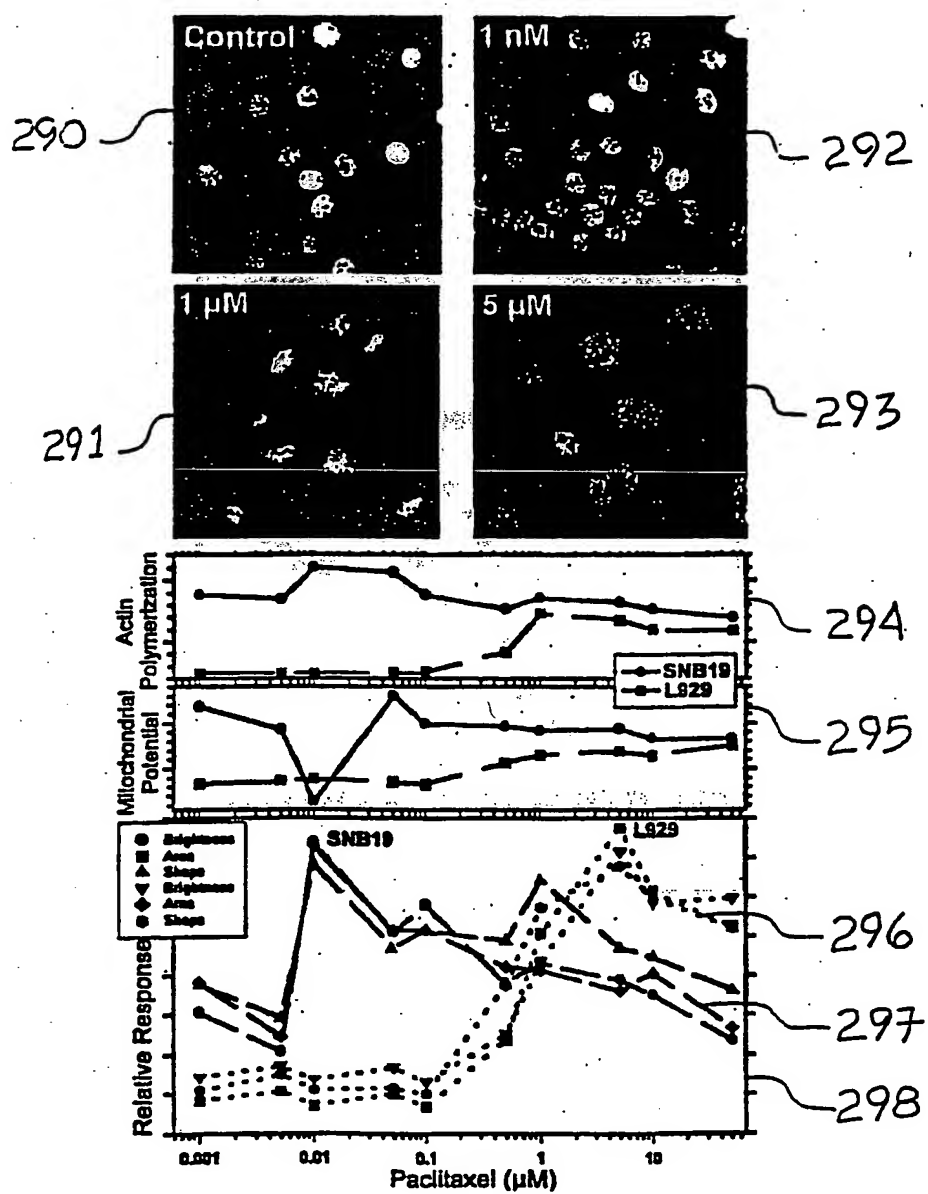


FIGURE 24

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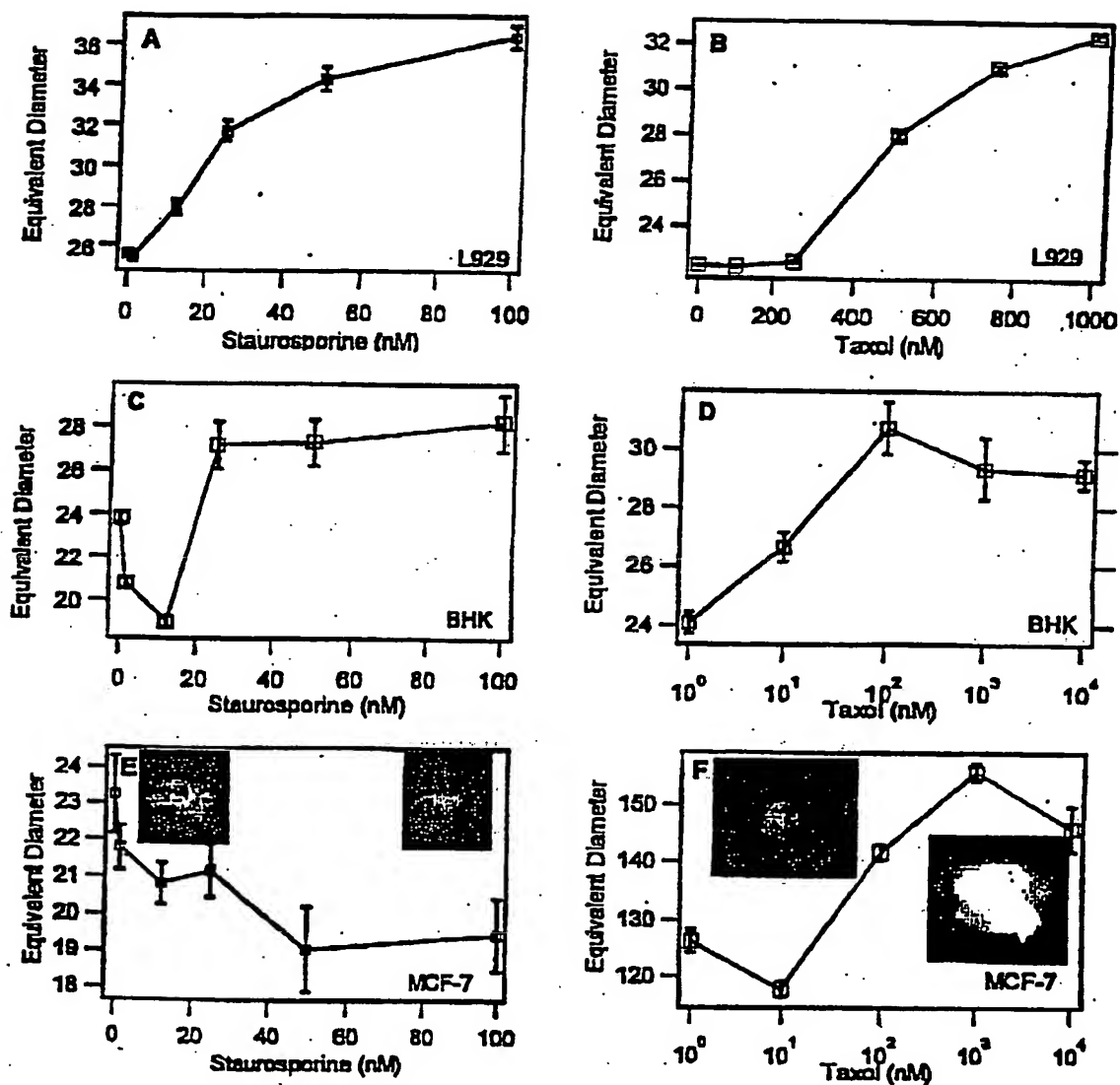
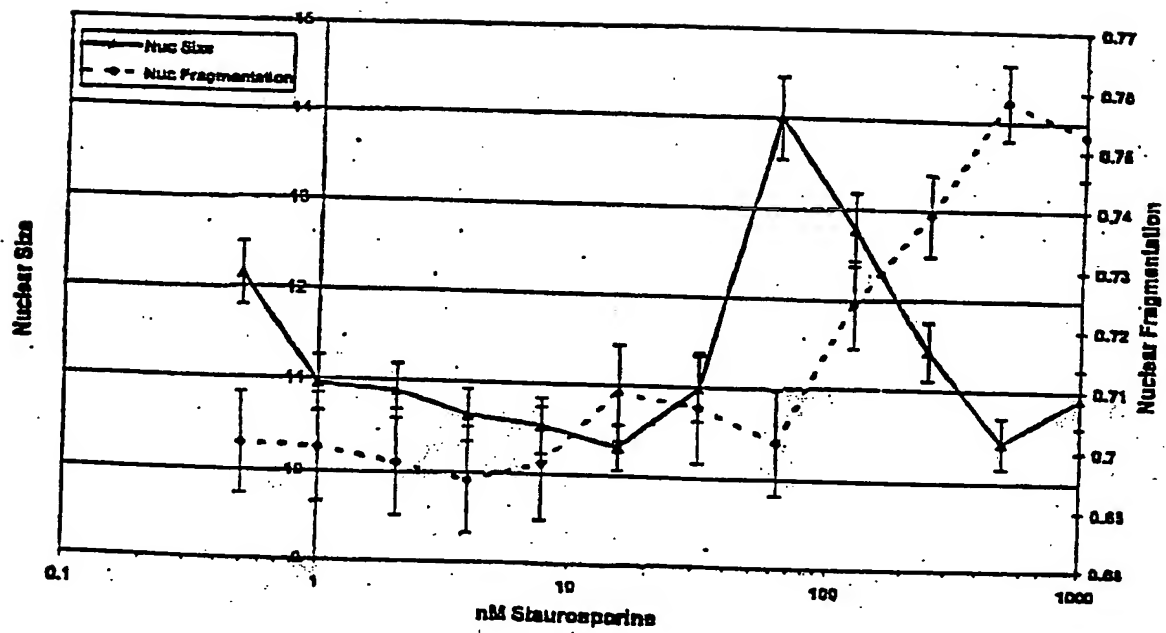


FIGURE 25

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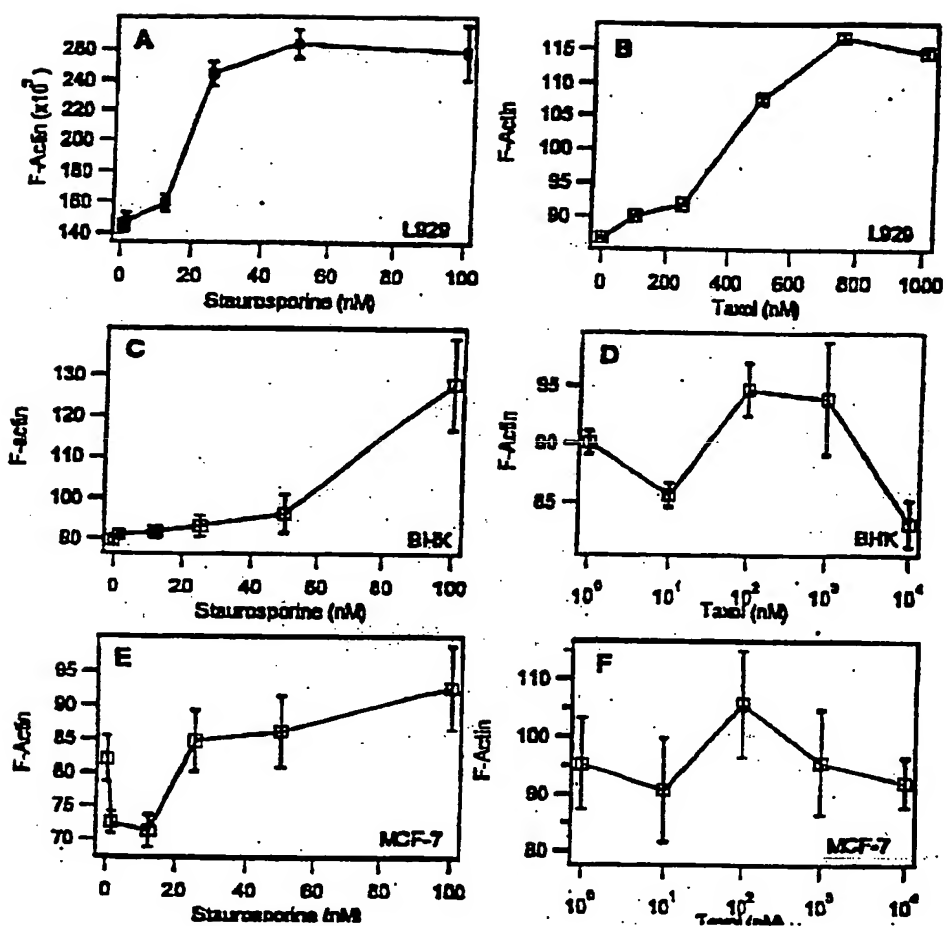


FIGURE 27

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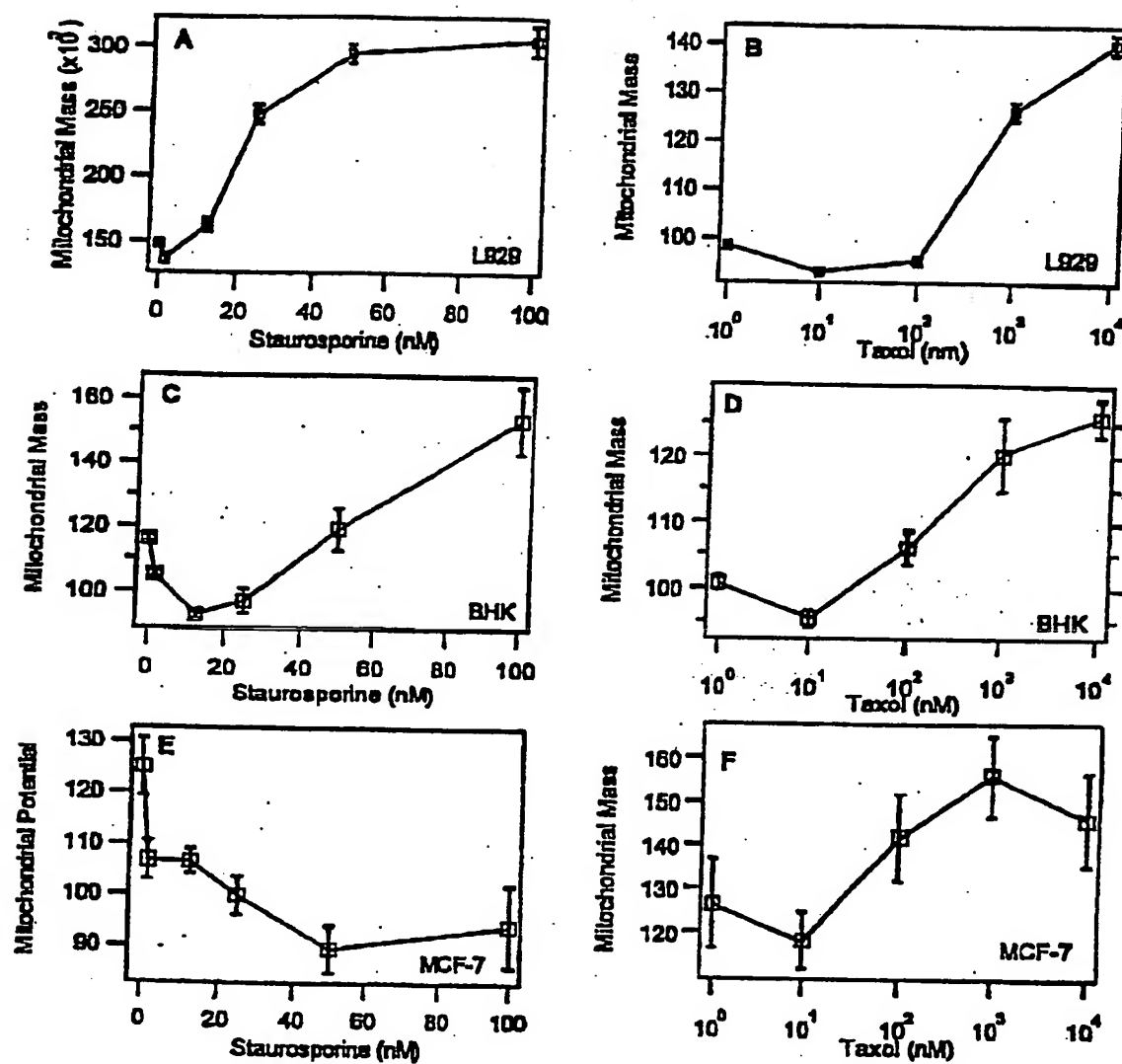


FIGURE 28



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## Mitochondrial Mass, Potential Data

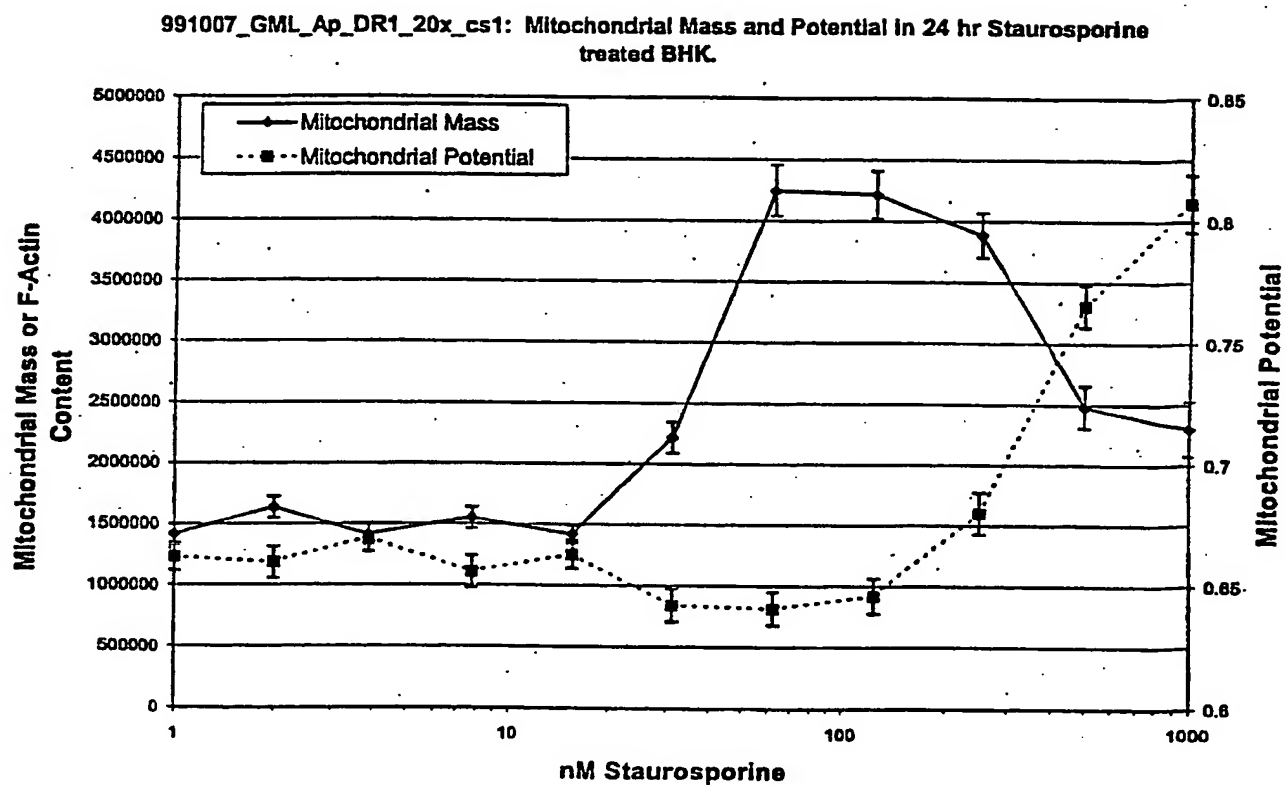


FIGURE 28G

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## 1. SIGNAL SEQUENCES

EPITOPE	SEQUENCE	SEQ ID NO:	REFERENCE
FLAG epitope	5' GACTACAAAGACGACG	35	Kasir, et al., 1999. J Biol Chem. 274:24873-80.
	AA Seq: ACGACAAA	36	
HA epitope	5' TACCCATACGACGTACCAGACTACGCA	37	Smith, et al., 1999. J Biol Chem. 274:19894-900.
	AA Seq: YPYDVPDYA	38	
KT3 epitope	5' CCACCAGAACCAGAAACA	39	MacArthur and Walter. 1984. J Virol. 52:483-91.
	AA seq: PPEPET	40	
Myc epitope	5' GCAGAAGAACAATAAATAAGCGAAGA AGACTTA	41	Gosney, et al., 1990. Anticancer Res. 10:623-8.
	AA Seq: AEEQKLISEEDL	42	

EYFP: SEQ ID NO: 43 (Nucleic acid); SEQ ID NO:44 (Amino acid)

M V S K G E E L F T G V V P I L V E L D  
 ATGGTGAGCAAG GCGGAGGAGCTG TTCACCGGGGTG GTGCCCATCCTG GTCGAGCTGGAC  
  
 G D V N G H K F S V S G E G E G D A T Y  
 GCGCAGCTAAAC GGCCACAAGTTC AGCGTGTCCGGC GAGGGCGAGGGC GATGCCACCTAC  
  
 G K L T L K F I C T T G K L P V P W P T  
 GGCAAGCTGACC CTGAAGTTCATC TGCACCACCGGC AAGCTGCCCCGTG CCCTGGCCCCACC  
  
 L V T T F G Y G L Q C F A R Y P D H M K  
 CTCGTGACCACC TTCGGCTACGGC CTGCAGTGCTTC GCCCGCTACCCC GACCACATGAAG  
  
 Q H D F F K S A M P E G Y V Q E R T I F  
 CAGCAGGACTTC TTCAAGTCCGCC ATGCCCCGAAGGC TACGTCCAGGAG CGCACCATCTTC  
  
 F K D D G N Y K T R A E V K F E G D T L  
 TTCAAGGACGAC GGCAACTACAAG ACCCGCGCCGAG GTGAAGTTCGAG GGCGACACCCTG  
  
 V N R I E L K G I D F K E D G N I L G H  
 GTGAACCGCATC GAGCTGAAGGGC ATCGACTTCAAG GAGGACGGCAAC ATCCTGGGGCAC  
  
 K L E Y N Y N S H N V Y I M A D K Q K N  
 AAGCTGGAGTAC AACTACAACAGC CACAACGTCTAT ATCATGGCCGAC AAGCAGAAGAAC  
  
 G I K V N F K I R H N I E D G S V Q L A  
 GGCATCAAGGTG AACTTCAAGATC CGCCACAACATC GAGGACGGCAGC GTGCAGCTCGCC  
  
 D H Y Q Q N T P I G D G P V L L P D N H  
 GACCACTACCAG CAGAACACCCCC ATCGGCGACGGC CCCGTGCTGCTG CCCGACAACCAC

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Y L S Y Q S A L S K D P N E K R D H M V  
TACCTGAGCTAC CAGTCCGCCCTG AGCAAAGACCCC AACGAGAAGCGC GATCACATGGTC  
L L E F V T A A G I T L G M D E L Y K  
CTGCTGGAGTTC GTGACCGCCGCC GGGATCACTCTC GGCATGGACGAG CTGTACAAG

EGFP: SEQ ID NO:45 (Nucleic acid); SEQ ID NO:46 (Amino acid)

M V S K G E E L F T G V V P I L V E L D  
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G D V N G H K F S V S G E G E G D A T Y  
GGCGACGTAAAC GGCCACAAGTTC AGCGTGTCCGGC GAGGGCGAGGGC GATGCCACCTAC  
G K L T L K F I C T T G K L P V P W P T  
GGCAAGCTGACC CTGAAGTTCATC TGCACCACCGGC AAGCTGCCCCGTG CCCTGGCCCCACC  
L V T T L T Y G V Q C F S R Y P D H M K  
CTCGTGACCACC CTGACCTACGGC GTGCAGTGCTTC AGCCGCTACCCC GACCACATGAAG  
Q H D F F K S A M P E G Y V Q E R T I F  
CAGCAGACTTC TTCAAGTCCGCC ATGCCCGAAGGC TACGTCCAGGAG CGCACCATCTTC  
F K D D G N Y K T R A E V K F E G D T L  
TTCAAGGACGAC GGCAACTACAAG ACCCGCGCCGAG GTGAAGTTCGAG GGCGACACCCTG  
V N R I E L K G I D F K E D G N I L G H  
GTGAACCGCATC GAGCTGAAGGGC ATCGACTTCAAG GAGGACGGCAAC ATCCTGGGGCAC  
K L E Y N Y N S H N V Y I M A D K Q K N  
AAGCTGGAGTAC AACTACAACAGC CACAACGTCTAT ATCATGGCCGAC AAGCAGAAGAAC  
G I K V N F K I R H N I E D G S V Q L A  
GGCATCAAGGTG AACTTCAAGATC CGCCACAACATC GAGGACGGCAGC GTGCAGCTCGCC  
D H Y Q Q N T P I G D G P V L L P D N H  
GACCACTACCAG CAGAACACCCCC ATCGGCGACGGC CCCGTGCTGCTG CCCGACAACCAC  
Y L S T Q S A L S K D P N E K R D H M V  
TACCTGAGCACC CAGTCCGCCCTG AGCAAAGACCCC AACGAGAAGCGC GATCACATGGTC  
L L E F V T A A G I T L G M D E L Y K  
CTGCTGGAGTTC GTGACCGCCGCC GGGATCACTCTC GGCATGGACGAG CTGTACAAG

EBFP: SEQ ID NO:47 (Nucleic acid); SEQ ID NO:48 (Amino acid)

M V S K G E E L F T G V V P I L V E L D  
ATGGTGAGCAAG GGCGAGGAGCTG TTCACCGGGGTG GTGCCCATCCTG GTCGAGCTGGAC

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G D V N G H K F S V S G E G E G D A T Y  
GGCGACGTAAAC GGCCACAAGTTC AGCGTGTCCGGC GAGGGCGAGGGC GATGCCACCTAC

G K L T L K F I C T T G K L P V P W P T  
GGCAAGCTGACC CTGAAGTTCATC TGCACCACCGGC AAGCTGCCCCGTG CCCTGGCCCCACC

L V T T L T H G V Q C F S R Y P D H M K  
CTCGTGACCACC CTGACCCACGGC GTGCAGTCTTC AGCCGCTACCCC GACCACATGAAG

Q H D F F K S A M P E G Y V Q E R T I F  
CAGCAGGACTTC TTCAAGTCCGCC ATGCCCGAAGGC TACGTCCAGGAG CGCACCATCTTC

F K D D G N Y K T R A E V K F E G D T L  
TTCAAGGACGAC GGCAACTACAAG ACCCGCGCCGAG GTGAAGTTCGAG GGCGACACCCTG

V N R I E L K G I D F K E D G N I L G H  
GTGAACCGCATC GAGCTGAAGGGC ATCGACTTCAAG GAGGACGGCAAC ATCCTGGGGCAC

K L E Y N F N S H N V Y I M A D K Q K N  
AAGCTGGAGTAC AACTTCAACAGC CACAACGTCTAT ATCATGGCCGAC AAGCAGAAGAAC

G I K V N F K I R H N I E D G S V Q L A  
GGCATCAAGGTG AACTTCAAGATC CGCCACAACATC GAGGACGGCAGC GTGCAGCTCGCC

D H Y Q Q N T P I G D G P V L L P D N H  
GACCACTACCAG CAGAACACCCCC ATCGGCGACGGC CCCGTGCTGCTG CCCGACAACCAC

Y L S T Q S A L S K D P N E K R D H M V  
TACCTGAGCACC CAGTCCGCCCTG AGCAAAGACCCC AACGAGAAGCGC GATCACATGGTC

L L E F V T A A G I T L G M D E L Y K  
CTGCTGGAGTTC GTGACCGCCGCC GGGATCACTCTC GGCATGGACGAG CTGTACAAG

ECFP: SEQ ID NO:49 (Nucleic acid); SEQ ID NO:50 (Amino acid)

M V S K G E E L F T G V V P I L V E L D  
ATGGTGAGCAAG GGCGAGGAGCTG TTCACCGGGGTG GTGCCCATCCTG GTCGAGCTGGAC

G D V N G H K F S V S G E G E G D A T Y  
GGCGACGTAAAC GGCCACAAGTTC AGCGTGTCCGGC GAGGGCGAGGGC GATGCCACCTAC

G K L T L K F I C T T G K L P V P W P T  
GGCAAGCTGACC CTGAAGTTCATC TGCACCACCGGC AAGCTGCCCCGTG CCCTGGCCCCACC

L V T T L T W G V Q C F S R Y P D H M K  
CTCGTGACCACC CTGACCTGGGGC GTGCAGTGCTTC AGCCGCTACCCC GACCACATGAAG

Q H D F F K S A M P E G Y V Q E R T I F  
CAGCAGGACTTC TTCAAGTCCGCC ATGCCCGAAGGC TACGTCCAGGAG CGCACCATCTTC

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F K D D G N Y K T R A E V K F E G D T L  
TTCAAGGACGAC GGCAACTACAAG ACCCGCGCCGAG GTGAAGTTCGAG GGCGACACCCTG  
V N R I E L K G I D F K E D G N I L G H  
GTGAACCGCATC GAGCTGAAGGGC ATCGACTTCAAG GAGGACGGCAAC ATCCTGGGGCAC  
K L E Y N Y I S H N V Y I T A D K Q K N  
AAGCTGGAGTAC AACTACATCAGC CACAACGTCTAT ATCACCGCCGAC AAGCAGAAGAAC  
G I K A N F K I R H N I E D G S V Q L A  
GGCATCAAGGCC AACTTCAAGATC CGCCACAACATC GAGGACGGCAGC GTGCAGCTCGCC  
D H Y Q Q N T P I G D G P V L L P D N H  
GACCACTACCAG CAGAACACCCCC ATCGGCGACGGC CCCGTGCTGCTG CCCGACAACCAC  
Y L S T Q S A L S K D P N E K R D H M V  
TACCTGAGCACC CAGTCCGCCCTG AGCAAAGACCCC AACGAGAAGCGC GATCACATGGTC  
L L E F V T A A G I T L G M D E L Y K  
CTGCTGGAGTTC GTGACCGCCGCC GGGATCACTCTC GGCATGGACGAG CTGTACAAG

**Fred25: SEQ ID NO:51 (Nucleic acid); SEQ ID NO:52 (Amino acid)**

M A S K G E E L F T G V V P I L V E L D  
ATGGCTAGCAAA GGAGAAGAACTC TTCACTGGAGTT GTCCCAATTCTT GTTGAATTAGAT  
G D V N G H K F S V S G E G E G D A T Y  
GGTGATGTAAAC GGCCACAAGTTC TCTGTCACTGGA GAGGGTGAAGGT GATGCAACATAC  
G K L T L K F I C T T G K L P V P W P T  
GGAAACTTACC CTGAAGTTCATC TGCACTACTGGC AAAGTGCCTGTT CCATGGCCAACA  
L V T T L C Y G V Q C F S R Y P D H M K  
CTAGTCACTACT CTGTGCTATGGT GTTCAATGCTTT TCAAGATACCCG GATCATATGAAA  
R H D F F K S A M P E G Y V Q E R T I F  
CGGCATGACTTT TTCAAGAGTGCC ATGCCCAGAGGT TATGTACAGGAA AGGACCATCTTC  
F K D D G N Y K T R A E V K F E G D T L  
TTCAAAGATGAC GGCAACTACAAG ACACGTGCTGAA GTCAAGTTTGAA GGTGATACCCTT  
V N R I E L K G I D F K E D G N I L G H  
GTTAATAGAATC GAGTTAAAAGGT ATTGACTTCAAG GAAGATGGCAAC ATTCTGGGACAC  
K L E Y N Y N S H N V Y I M A D K Q K N  
AAATTGGAATAC AACTATAACTCA CACAATGTATAC ATCATGGCAGAC AAACAAAAGAAT  
G I K V N F K T R H N I E D G S V Q L A  
GGAATCAAAGTG AACTTCAAGACC CGCCACAACATT GAAGATGGAAGC GTTCAACTAGCA  
D H Y Q Q N T P I G D G P V L L P D N H

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GACCATTATCAA CAAAATACTCCA ATTGGCGATGGC CCTGTCCTTTTA CCAGACAACCAT

Y L S T Q S A L S K D P N E K R D H M V  
TACCTGTCCACA CAATCTGCCCTT TCGAAAGATCCC AACGAAAAGAGA GACCACATGGTC

L L E F V T A A G I T H G M D E L Y N \*  
CTTCTTGAGTTT GTAACAGCTGCT GGGATTACACAT GGCATGGATGAA CTGTACAACCTAG

## 2. PROTEASE RECOGNITION SITES

Substrate Recognitions Sequences	Source	Recognition Site	SEQ ID NO	Reference
Caspase-1,4,5	peptide library	5'(TGG,TTA)GAACATGACAA Seq:(W,L)EHD/	53 54	Thornberry et al., 1997, J. Biol. Chem. 272:17907
proCaspase-1	peptide library	5'TGGTTTAAAGAC AA Seq: WFKD/	55 56	Thornberry et al., 1997, J. Biol. Chem. 272:17907
Caspase-2	peptide library	5'GACGAACAC'GAC AA Seq: DEHD/	57 58	Thornberry et al., 1997, J. Biol. Chem. 272:17907
Caspase 3, 7	PARP	5'GACGAAGTTGAC AA Seq: DEVG/	59 60	Beneke, et al., 1997, Biochem Mol Biol Int. 43:755-61; Thornberry et al., 1997, J. Biol. Chem. 272:17907
ProCaspase 3	Caspase-3	5'ATAGAAACAGAC AA Seq: IETD/	61 62	Tewari, M., et al., 1995, Cell. 81:801-9.
ProCaspase-4,5	peptide library	5'TGGGTAAGAGAC AA Seq: WVRD/	63 64	Thornberry, N.A. et al., 1997, J. Biol. Chem. 272, 17907-17911
Caspase 6	Lamin A, peptide library	5'GTAGAAATAGAC AA Seq: VEID/ 5'GTAGAACACGAC AA Seq: VEHD/	65 66 67 68	Nakajima and Sado. 1993. Biochim Biophys Acta. 1171:311-4; Thornberry et al., 1997, J. Biol. Chem. 272:17907
proCaspase 6	Caspase-6	5'ACAGAAAGTAGAC AA Seq: TEVD/	69 70	Fernandes-Alnemri, et al., 1994, J Biol Chem. 269:30761-4.
proCaspase-7	peptide library	5'ATACAAGCAGAC AA Seq: IQAD/	71 72	Thornberry, N.A. et al., 1997, J. Biol. Chem. 272, 17907-17911
Caspase 8	peptide library	5'GTAGAAACAGAC AA Seq: VETD/	73 74	Muzio, M., et al., 1996, Cell. 85:817-27; Fernandes-Alnemri, et al., 1996, Proc Natl Acad Sci U S A. 93:7464-9; Thornberry et al., 1997, J. Biol. Chem. 272:17907
proCaspase-8	Caspase-8	5'TTAGAAACAGAC AA Seq: LETD/	75 76	Muzio, M., et al., 1996, Cell. 85:817-27; Fernandes-Alnemri, et al., 1996, Proc Natl Acad Sci U S A. 93:7464-9; Thornberry et al., 1997, J. Biol. Chem. 272:17907
Caspase 9	peptide library	5'TTAGAACACGAC AA Seq: LEHD/	77 78	Thornberry, N.A. et al., 1997, J. Biol. Chem. 272, 17907-17911
proCaspase 9	Caspase-9	CCCGAACCCGAC PEPD	79 80	Thornberry, N.A. et al., 1997, J. Biol. Chem. 272, 17907-17911
HIV protease		5'AGCCAAAATTAC AA Seq: SQNY/ 5'CCAATAGTACAA AA Seq: PIVQ/	81 82 83 84	Matayoshi, et al., 1990, Science. 247:954-8.
Adenovirus endopeptidase		5'AUGTTTGGAGGA AA Seq: MFGG/ 5'GCAAAAAAAGA AA Seq: AKKR/	85 86 87 88	Weber and Tihanyi. 1994. Methods Enzymol. 244:595-604.
b-Secretase	Amyloid precursor protein	5'GTAAAAAUG AA Seq: VKM/ 5'GACGCAGAATTC DAEF/	89 90 91 92	Hardy et al., 1994, in Amyloid Protein Precursor in Development, Aging, and Alzheimer's Disease, ed. C.L. Masters et al., pp. 190-198.
Cathepsin D		5'AAACCAGCATTATTC AA Seq: KPALF 5'TTCAGATTA AA Seq: FRL/	93 94 95 96	Dunn, et al., 1998. Adv Exp Med Biol. 436:133-8.
Matrix Metalloproteases		5'GGACCATTAGGACCA AA Seq: GPLGP	97 98	Bouvier et al., 1993; Garbett et al., 1999; Hill and Sakanari, 1997;

				Kojima et al., 1998; Tyagi et al., 1995; Wilhelm et al., 1993; Williams and Auld, 1986; Haugland, R., Handbook of fluorescent probes and research Chemicals 7th ed.
Granzyme B	peptide library	5'ATAGAACCAGAC AA Seq: IEPD/	99 100	Thornberry et al., 1997, J. Biol. Chem. 272:17907
Anthrax protease	MEK1	5'ATGCCCAAGAAGAAGCCGAC GCCCATCCAGCTGAACCC  AA Seq: MPKKKPTPIQLN	101  102	Vitale et al., (1998) Biochem Biophys Res Commun 248 (3), 706-711
Anthrax protease	MEK2	5'ATGCTGGCCCGGAGGAAGCCG GTGCTGCCGGCGCTCACCATCA ACCC  AA Seq: MLARRKPVLPAITIN	103  104	Vitale et al., (1998) Biochem Biophys Res Commun 248 (3), 706-711
tetanus/botulinum	cellubrevin	5'GCCTCGCAGTTTGAAACA  AA Seq: ASQFET	105  106	McMahon et al., Nature 364:346-349; Martin et al., J. Cell Biol. In press
tetanus/botulinum	synaptobrevin/ VAMP3	5'GCTTCTCAATTGAAACG  AA Seq: ASQFET	107  108	Schiavo et al., (1992) Nature 359, 832-5
Botulinum neurotoxin A	SNAP-25	5'GCCAACAACGTGCAACA AA Seq: ANQ/RAT	109 110	Zhao, et al. Gene 145 (2), 313-314 (1994)
Botulinum neurotoxin B	VAMP	5'GCTTCTCAATTGAAACG AA Seq: ASQ/FET	111 112	
Botulinum neurotoxin C	Syntaxin	5'ACGAAAAAAGCTGTGAAA AA Seq: TKK/AVK	113 114	Martin et al., J. Leukoc. Biol. 65 (3), 397-406 (1999)
Botulinum neurotoxin D	VAMP	5'GACCAGAAGCTCTCTGAG AA Seq: DQK/LSE	115 116	
Botulinum neurotoxin E	SNAP-25	5'ATCGACAGGATCATGGAG AA Seq: IDR/IME	117 118	
Botulinum neurotoxin F	VAMP	5'AGAGACCAGAAGCTCTCT AA Seq: RDO/KLS	119 120	
Botulinum neurotoxin G	VAMP	5'ACGAGCGCAGCCAAGTTG AA Seq: TSA/AKL	121 122	



## 3. PRODUCT/REACTANT TARGET SEQUENCES

Target	Target Source	Target domain (Product or Reactant)	SEQ ID NO	Reference
Cytoplasm/cytoskeleton	Annexin II	5'ATGTCTACTGTCCACGAAATCCTGTGCAAGCTCAGCTTGGAGGGTGTTCATTCTACACCCCCAAGTGCC 3'	123	Eberhard, et al., 1997, Mol. Biol. Cell 8:293a.
		(Amino acid seq: M S T V H E I L C K L S L E G V H S T P P S A)	124	
Inner surface of plasma membrane	farnesylation	5'AUGGGATCTACATTAAGCGCAGAAGACAAAGCAGCAGTAGAAAGAAGCAAAAUGATAGACAGAACTTATTAAGAGAAGACGGAGAAAAAGCTGCTAGA3'	125	Ferruccio G. et al., J. Biol. Chem. 274, 5843-5850, 1999
		(AA seq: M G C T L S A E D K A A V E R S K M I D R N L R E D G E K A A R)	126	
Nucleus	NFkB p50	5'AGAAGGAAACGACAAAAG (AA seq: R R K R Q K)	127 128	Henkel, T et al., Cell 68, 1121-1133, 1992
Nucleolus	NOLP	5'AGAAAACGTATACGTACTTACCTCAAGTCCGTCAGGCGGATGAAAAGAAGTGGTTTGAGATGCTCGACCTATTCCTTCCACCTTACT	129	Ueki, et al., 1998, Biochem Biophys Res Commun. 252:97-102.
		(AA seq: R K R I R T Y L K S C R R M K R S G F E M S R P I P S H L T)	130	
Mitochondria	cytochrome c oxidase	5'ATGTCCGTCTGACGCCGCTGCTGCTGCGGGCTTGACAGGCTCGGCCCGGCGGCTCCAGTGCCGCGCGCCAAGATCCATTCTGTTG	131	Rizzuto, et al., 1989, J Biol Chem. 264:10595-600.
		(AA Seq: M S V L T P L L L R G L T G S A R R L P V P R A L I H S L)	132	
Nuclear Envelope	ODV-E66 & ODV-E25	5'AUGAGCATTGTTTTAATAATTGTTATTGGA TTTTTTAATATGTTTTTATATTTAAGCAACAGCAAAGATCCCAGAGTACCAGTTGAATTAU G	133	Hong, T, et al. PNAS, 94, 4050-4055, 1997
		(AA Seq: M S I V L I I V I V I F L I C F L Y L S N S K D P R V P V E L M)	134	
Golgi	Calreticulin	5'ATGAGGCTTCGGGAGCCGCTCCTGAGCGGCAGCGCCGCGATGCCAGGCGGCTCCCTACAGCGGCCTGCCGCTGCTCGTGCCGCTCGCGCTCTGCACCTTGGCGTCAACCTCGTTACTACCTGGCTGGCCGCGACCTGAGCCGCTGCCCAA CTGGTCGGAGTCTCCACACCGCTGCAGGGCG GCTCGAACAGTGCCGCGGCCATCGGGCAGTC CTCCGGGGAGCTCCGGACCGGAGGGGCC	135	Fliegel, L., et al., J. Biol. Chem. 264, 21522-21528, 1989.
		(AA Seq: M R L R E P L L S G S A A M P G A S L Q R A C R L L V A V C A L H L G V T L V Y Y L A G R D L S R L P Q L V G V S T P L Q G S N S A A I G O S S Q E L R T G G A)	136	
Endoplasmic reticulum	D-AKAP1	5'GAAACAATAAGACCTATAAGAAGATGTAGTACATTACATCTACAGACAGCAAAAUGGCAATTCATTAAGATCTCCCTTTCCATTAGCATTA CCAGGAUGTTAGCTTTATTAGGATGGTGGTGGTTTTTCAGTAGAAAAAAA	137	Huang, L.J. Et al., J. Cell. Biol. 145, 951-959, 1999
		(AA Seq: E T I R P I R I R C S Y F T S T D S K M A I Q L R S P F P L A L P G M L A L L G W W F F S R K K)	138	
Nuclear Export	MEK1	5' GCCTTG CAGAAGAAGCTGGAGGAGCT AGAGCTTGATGAG	139	Fukuda, (1997) J. Biol. Chem

FIGURE 29C

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		(AA SEQ: A L Q K K L E E L E L D E	140	272, 51, 32642- 32648
Size exclusion	PROJ domain of MAP4	<p>5'GCCGACCTCAGTCTTGTGGATGCGTTFGACA GAACCACCTCCAGAAATTGAGGGAGAAATAA AGCGAGACTTCATGGCTGCGCTGGAGGCAQA GCCCTATGATGACATCGTGGGAGAACTGTG GAGAAAACTGAGTTTATTCCTCTCTCTGGATGG TGATGAGAAAAACCGGAACTCAGAGTCCAAA AAGAAACCCTGCTTAGACACTAGCCAGGTTG AAGGTATCCCATCTTCTAAACCAACACTCCTA GCCAATGGTOATCATGGAATGGAGGGGAATA ACACTGCAGGGTCTCCAAGTGAATTCCTTGAA GAGAGAGTGGACTATCCGGATTATCAGAGCA GCCAGAACTGGCCAGAAGATGCAAGCTTTTG TTTCCAGCTCAGCAAGTGTAGATACTGACC AGGCTGAGCCCTTAACGAGCACCCTGATGA TGGTTTGGCAGATCTGCTCTTTGTCTCCAGTG GACCCACGAACGCTTCTGCATTTACAGAGCG AGACAACTCTCAGAAGACAGTTACGGTATG CTTCCCTGTGACTCATTGTCTTCCAGGCTGT TGTATCTCAGGAGTGGTCTGTGGGAGCCCCA AACTCTCCATGTTTCAGAGTCTGTGTCTCCCC AGAGGTTACTATAGAAACCCCTACAGCCAGCA ACAGAGCTCTCCAAGGCAGCAGAAGTGGAAT CAGTGAAAGAGCAGCTGCCAGCTAAAGCATT GGAAACGATGGCAGAGCAGACCACTGATGTG GTGCACTCTCCATCCACAGACACAACACCAG GCCCAGACACAGAGGCAGCACTGGCTAAAGA CATAGAAGAGATCACCAAGCCAGATGTGATA TTGGCAAAATGTCACGCAGCCATCTACTGAAT CGGATATGTTCTGGCCAGGACATGGAAT ACTCAGAGGAACAGAGGCAGCCACGCTAAC AATATCATATTGCCTACAGAACCAGACGAAT CTTCAACCAAGGATGTAGCACCACCTATGGA AGAAGAAATTGTCCAGGCAATGATA</p> <p>(AA SEQ: A D L S L V D A L T E P P P E I E G E I K R D F M A A L E A E P Y D D I V G E T V E K T E F I P L L D G D E K T G N S E S K K K P C L D T S Q V E G I P S S K P T L L A N G D H G M E G N N T A G S P T D F L E E R V D Y P D Y Q S S Q N W P E D A S F C F Q P Q V L D T D Q A E P F N E H R D D G L A D L L F V S S G P T N A S A F T E R D N P S E D S Y G M L P C D S F A S T A V V S Q E W S V G A P N S P C S E S C V S P E V T I E T L Q P A T E L S K A A E V E S V K E Q L P A K A L E T M A E Q T T D V V H S P S T D T T P G P D T E A A L A K D I E E I T K P D V I L A N V T Q P S T E S D M F L A Q D M E L L T G T E A A H A N N I I L P T E P D E S S T K D V A P P M E E E I V P G N D T T S P K E T E T T L P I K M D L A P P E D V L L T K E T E L A P A K G M V S L S E I E E A L A K N D V R S A E I P V A Q E T V V S E T E V V L A T E V V L P S D P I T T L T K D V T L P L E A E R P L V T D M T P S L E T E M T L G K E T A P P T E T N L G M A K D M S P L P E S E V T L G K D V V I L P E T K V A E F N N V T P L S E E E V T S V K D M S P S A E T E A P L A K N A D L H S G T E L I V D N S M A P A S D L A L P L E T K V A T V P I K D K G</p>	141	West, (1991). J Biol Chem 266(32): 21886- 96; Olson, K. R. (1995). J Cell Biol 130(3): 639- 50.
			142	
Vesicle membrane	Synaptobrevin	<p>5' ATGTGGGCAATCGGGATTACTGTTCT GGTTATCTTCATCATCATCATCATCGTG TGGGTTGTC</p> <p>(AA SEQ: M W A I G I T V L V I F I I I I I V W V V)</p>	143	Schiavo et al., (1992) Nature 359, 832-5
			144	

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Vesicle membrane	Cellubrevin	5' ATGTGGGCGATAGGGATCAGTGTCTT GGTGATCATTGTATCATCATCATCGTG TGGTGTG	145	McMahon et al., Nature 364:346- 349; Martin et al., J. Cell Biol. In press
		(AA SEQ: M W A I G I S V L V I I V I I I I V W C)	146	
Nuclear Export	MEK2	5' GACCTGCAGAAGAAGCTGGAGGAGCT GGAACCTTGACGAG	147	Zheng and Guan, J. Biol. Chem. 268:11435-11439, 1993
		AA SEQ: D L Q K K L E E L E L D E	148	
Peroxisome	PX	5' TCTAAACTG	149	Amery et al., Biochem. J. 336:367-371 (1998)
		AA SEQ: S K L	150	

Microtubules (MAP4) SEQ ID NO:151 (Nucleic acid); SEQ ID NO:152 (amino acid)

MAP4:

M A D L S L V D A L T E P P P E I E G E  
 ATGGCCGACCTC AGTCTTGTGGAT GCGTTGACAGAA CCACCTCCAGAA ATTGAGGGAGAA  
 TACCGGCTGGAG TCAGAACACCTA CGCAACTGTCTT GGTGGAGGTCTT TAACTCCCTCTT  
  
 I K R D F M A A L E A E P Y D D I V G E  
 ATAAAGCGAGAC TTCATGGCTGCG CTGGAGGCAGAG CCCTATGATGAC ATCGTGGGAGAA  
 TATTTTCGCTCTG AAGTACCGACGC GACCTCCGTCTC GGGATACTACTG TAGCACCCTCTT  
  
 T V E K T E F I P L L D G D E K T G N S  
 ACTGTGGAGAAA ACTGAGTTTATT CCTCTCCTGGAT GGTGATGAGAAA ACCGGGAACCTCA  
 TGACACCTCTTT TGA CTCAAATAA GGAGAGGACCTA CCACTACTCTTT TGGCCCTTGAGT  
  
 E S K K K P C L D T S Q V E G I P S S K  
 GAGTCCAAAAAG AAACCCTGCTTA GACACTAGCCAG GTTGAAGGTATC CCATCTTCTAAA  
 CTCAGGTTTTTC TTTGGGACGAAT CTGTGATCGGTC CAACTTCCATAG GGTAGAAGATTT  
  
 P T L L A N G D H G M E G N N T A G S P  
 CCAACACTCCTA GCCAATGGTGAT CATGGAATGGAG GGGAATAACACT GCAGGGTCTCCA  
 GGTGTGAGGAT CGGTTACCACTA GTACCTTACCTC CCCTTATTGTGA CGTCCCAGAGGT  
  
 T D F L E E R V D Y P D Y Q S S Q N W P  
 ACTGACTTCCTT GAAGAGAGAGTG GACTATCCGGAT TATCAGAGCAGC CAGAACTGGCCA  
 TGACTGAAGGAA CTTCTCTCTCAC CTGATAGGCCTA ATAGTCTCGTCG GTCTTGACCGGT  
  
 E D A S F C F Q P Q Q V L D T D Q A E P  
 GAAGATGCAAGC TTTTGTTCCTCAG CCTCAGCAAGTG TTAGATACTGAC CAGGCTGAGCCC  
 CTTCTACGTTTCG AAAACAAAGGTC GGAGTCGTTTAC AATCTATGACTG GTCCGACTCGGG  
  
 F N E H R D D G L A D L L F V S S G P T  
 TTTAACGAGCAC CGTGATGATGGT TTGGCAGATCTG CTCTTTGTCTCC AGTGACCCACG  
 AAATTGCTCGTG GCACTACTACCA AACCCTCTAGAC GAGAAACAGAGG TCACCTGGGTGC  
  
 N A S A F T E R D N P S E D S Y G M L P  
 AACGCTTCTGCA TTTACAGAGCGA GACAATCCTTCA GAAGACAGTTAC GGTATGCTTCCC  
 TTGCGAAGACGT AAATGTCTCGCT CTGTTAGGAAGT CTTCTGTCAATG CCATACGAAGGG

C D S F A S T A V V S Q E W S V G A P N  
TGTGACTCATTT GCTTCCACGGCT GTTGTATCTCAG GAGTGGTCTGTG GGAGCCCCAAAC  
ACACTGAGTAAA CGAAGGTGCCGA CAACATAGAGTC CTCACCAGACAC CCTCGGGGGTTTG

S P C S E S C V S P E V T I E T L Q P A  
TCTCCATGTTCA GAGTCCTGTGTC TCCCCAGAGGTT ACTATAGAAACC CTACAGCCAGCA  
AGAGGTACAAGT CTCAGGACACAG AGGGGTCTCCAA TGATATCTTTGG GATGTCGGTTCGT

T E L S K A A E V E S V K E Q L P A K A  
ACAGAGCTCTCC AAGGCAGCAGAA GTGGAATCAGTG AAAGAGCAGCTG CCAGCTAAAGCA  
TGTCTCGAGAGG TTCCGTCGTCTT CACCTTAGTCAC TTTCTCGTCGAC GGTCGATTTTCGT

L E T M A E Q T T D V V H S P S T D T T  
TTGGAAACGATG GCAGAGCAGACC ACTGATGTGGTG CACTCTCCATCC ACAGACACAACA  
AACCTTTGCTAC CGTCTCGTCTGG TGA CTACACCAC GTGAGAGGTAGG TGTCTGTGTTGT

P G P D T E A A L A K D I E E I T K P D  
CCAGGCCAGAC ACAGAGGCAGCA CTGGCTAAAGAC ATAGAAGAGATC ACCAAGCCAGAT  
GGTCCGGGTCTG TGTCTCCGTCGT GACCGATTTCTG TATCTTCTCTAG TGGTTCCGGTCTA

V I L A N V T Q P S T E S D M F L A Q D  
GTGATATTGGCA AATGTCACGCAG CCATCTACTGAA TCGGATATGTTT CTGGCCCAGGAC  
CACTATAACCGT TTACAGTCCGTC GGTAGATGACTT AGCCTATACAAG GACCGGGTCTCTG

M E L L T G T E A A H A N N I I L P T E  
ATGGAATACTC ACAGGAACAGAG GCAGCCCACGCT AACAATATCATA TTGCCTACAGAA  
TACCTTGATGAG TGTCTTGTCTC CGTCGGGTGCCA TTGTTATAGTAT AACGGATGTCTT

P D E S S T K D V A P P M E E E I V P G  
CCAGACGAATCT TCAACCAAGGAT GTAGCACCACCT ATGGAAGAAGAA ATTGTCCCAGGC  
GGTCTGCTTAGA AGTTGGTTCCTA CATCGTGGTGGA TACCTTCTTCTT TAACAGGGTCCG

N D T T S P K E T E T T L P I K M D L A  
AATGATACGACA TCCCCAAAGAA ACAGAGACAACA CTTCCAATAAAA ATGGACTTGGCA  
TTACTATGCTGT AGGGGGTTTCTT TGTCTCTGTTGT GAAGGTTATTTT TACCTGAACCGT

P P E D V L L T K E T E L A P A K G M V  
CCACCTGAGGAT GTGTTACTTACC AAAGAAACAGAA CTAGCCCCAGCC AAGGGCATGGT  
GGTGGACTCCTA CACAATGAATGG TTTCTTTGTCTT GATCGGGGTCCG TTCCCGTACCAA

S L S E I E E A L A K N D V R S A E I P  
TCACTCTCAGAA ATAGAAGAGGCT CTGGCAAAGAAT GATGTTCTGCTCT GCAGAAATACCT  
AGTGAGAGTCTT TATCTTCTCCGA GACCGTTTCTTA CTACAAGCGAGA CGTCTTTATGGA

V A Q E T V V S E T E V V L A T E V V L  
GTGGCTCAGGAG ACAGTGGTCTCA GAAACAGAGGTG GTCCTGGCAACA GAAGTGGTACTG  
CACCGAGTCCTC TGTCAACAGAGT CTTTGTCTCCAC CAGGACCGTTGT CTTCAACCATGAC

P S D P I T T L T K D V T L P L E A E R  
CCCTCAGATCCC ATAACAACATTG ACAAAGGATGTG ACACTCCCCTTA GAAGCAGAGAGA  
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A P P T E T N L G M A K D M S P L P E S  
GCTCCACCCACA GAAACAAATTTG GGCATGGCCAAA GACATGTCTCCA CTCCCAGAATCA  
CGAGGTGGGTGT CTTTGTTTAAAC CCGTACCGGTTT CTGTACAGAGGT GAGGGTCTTAGT

E V T L G K D V V I L P E T K V A E F N  
GAAGTGACTCTG GGCAAGGACGTG GTTATACTTCCA GAAACAAAGGTG GCTGAGTTTAAAC  
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N V T P L S E E E V T S V K D M S P S A  
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E T E A P L A K N A D L H S G T E L I V  
GAAACAGAGGCT CCCCTGGCTAAG AATGCTGATCTG CACTCAGGAACA GAGCTGATTGTG  
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D N S M A P A S D L A L P L E T K V A T  
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V P I K D K G T V Q T E E K P R E D S Q  
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L A S M Q H K G Q S T V P P C T A S P E  
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AATCGTAGATAC GTCGTGTTCCCT GTCAGTTGTCAT GGAGGAACGTGC CGAAGTGGTCTT

P V K A A E Q M S T L P I D A P S P L E  
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TTGAATCTCGTC TTCCTTTGCGGA CCGTCGGTCGGA AGACTCGGAACG AGTCCTCATAGG

R Q E E A K A A V G V T G N D I T T P P  
CGGCAAGAAGAA GCAAAGGCTGCT GTAGGTGTGACT GGAAATGACATC ACTACCCCGCCA  
GCCGTTCTTCTT CGTTTCCGACGA CATCCACACTGA CCTTACTGTAG TGATGGGGCGGT

N K E P P P S P E K K A K P L A T T Q P  
AACAAGGAGCCA CCACCAAGCCCA GAAAAGAAAGCA AAGCCTTTGGCC ACCACTCAACCT  
TTGTTCTCTCGGT GGTGGTTTCGGGT CTTTCTTTTCGT TTCGAAACCGG TGGTGAGTTGGA

A K T S T S K A K T Q P T S L P K Q P A  
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A A P H K R P A A A T A T A R P S T L P  
GCTGCCCCACAC AAACGCCCTGCT GCTGCCACTGCT ACTGCCAGGCCT TCCACCCTACCT  
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A R D V K P K P I T E A K V A E K R T S  
GCCAGAGACGTG AAGCCAAAGCCA ATTACAGAAGCT AAGGTTGCCGAA AAGCGGACCTCT  
CGGTCTCTGCAC TTCGGTTTCGGT TAATGTCTTCGA TTCCAACGGCTT TTCGCCTGGAGA

P S K P S S A P A L K P G P K T T P T V  
CCATCCAAGCCT TCATCTGCCCA GCTCTCAAACCT GGACCTAAAACC ACCCCAACCGTT  
GGTAGGTTCCGA AGTAGACGGGT CGGGAGTTTGGA CCTGGATTITGG TGGGGTTGGCAA

S K A T S P S T L V S T G P S S R S P A  
TCAAAAGCCACA TCTCCCTCAACT CTTGTTTCCACT GGACCAAGTAGT AGAAGTCCAGT  
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T T L P K R P T S I K T E G K P A D V K  
ACAACTCTGCCT AAGAGGCCAACC AGCATCAAGACT GAGGGGAAACCT GCTGATGTCAAA  
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R M T A K S A S A D L S R S K T T S A S  
AGGATGACTGCT AAGTCTGCCTCA GCTGACTTGAGT CGCTCAAAGACC ACCTCTGCCAGT  
TCCTACTGACGA TTCAGACGGAGT CGACTGAACTCA GCGAGTTTCTGG TGGAGACGGTCA

S V K R N T T P T G A A P P A G M T S T  
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AGACACTTCTCT TTGTGGTGAGGG TGACCCCGTCGT GGGGGTCGTCCC TACTGAAGGTGA

R V K P M S A P S R S S G A L S V D K K  
CGAGTCAAGCCC ATGTCTGCACCT AGCCGCTCTTCT GGGGCTCTTTCT GTGGACAAGAAG  
GCTCAGTTCCGG TACAGACGTGGA TCGGCGAGAAGA CCCCAGAGAAAGA CACCTGTTCTTC

P T S T K P S S S A P R V S R L A T T V  
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GGGTGAAGGTGA TTCGGATCGAGG AGACGAGGGTCC CACTCGGCGGAC CGGTGTTGACAA

S A P D L K S V R S K V G S T E N I K H  
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AGACGGGGACTG GACTTCTCACAA GCGAGGTTCCAG CCGAGATGTCTT TTGTAGTTTGTG

Q P G G G R A K V E K K T E A A T T A G  
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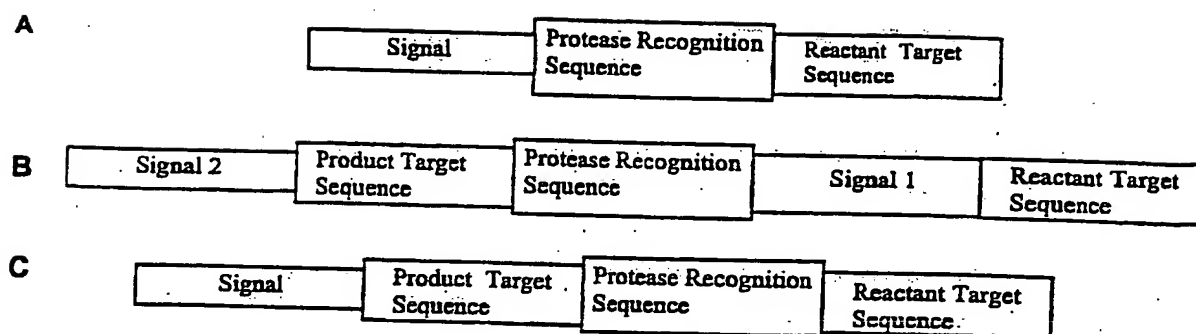
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C V S K D N I K H V P G C G N V Q I Q N  
TGTGTTTCCAAG GACAATATTAAG CATGTCCCTGGA TGTGGCAATGTT CAGATTCAGAAC  
ACACAAAGGTTC CTGTTATAATTC GTACAGGGACCT ACACCGTTACAA GTCTAAGTCTTG  
K K V D I S K V S S K C G S K A N I K H  
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TTCTTTCACCTG TATAGGTTCCAG AGGAGGTTTACA CCCAGGTTTCGA TTATAGTTTCGTG  
K P G G G D V K I E S Q K L N F K E K A  
AAGCCTGGTGGG GGAGATGTCAAG ATTGAAAGTCAG AAGTTGAACCTC AAGGAGAAGGCC  
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Q A K V G S L D N V G H F P A G G A V K  
CAAGCCAAAGTG GGATCCCTTGAT AACGTTGGCCAC TTTCTGTCAGGA GGTGCCGTGAAG  
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T E G G G S E A L P C P G P P A G E E P  
ACTGAGGGCGGT GGCAGTGAGGCC CTTCCGTGTCCA GGGCCCCCGCT GGGGAGGAGCCA  
TGACTCCCGCCA CCGTCACTCCGG GAAGGCACAGGT CCGGGGGGGCGA CCCCTCCTCGGT  
V I P E A A P D R G A P T S A S G L S G  
GTCATCCCTGAG GCTGCGCCTGAC CGTGGCGCCCCT ACTTCAGCCAGT GGCCTCAGTGGC  
CAGTAGGGACTC CGACGCGGACTG GCACCGCGGGGA TGAAGTCGGTCA CCGGAGTCACCG  
H T T L S G G G D Q R E P Q T L D S Q I  
CACACCACCTG TCAGGGGGTGGT GACCAAAGGGAG CCCCAGACCTTG GACAGCCAGATC  
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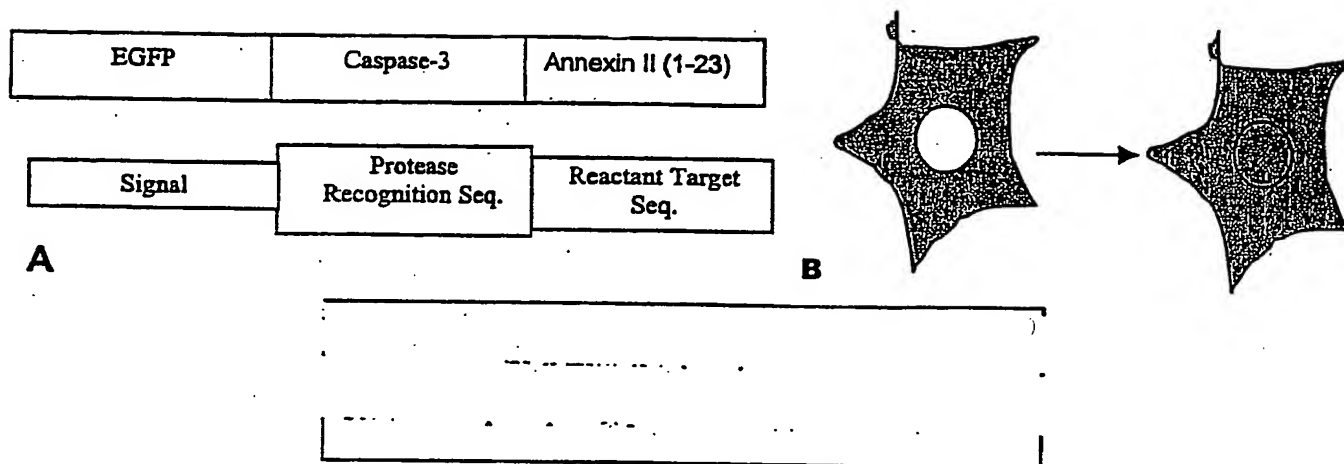
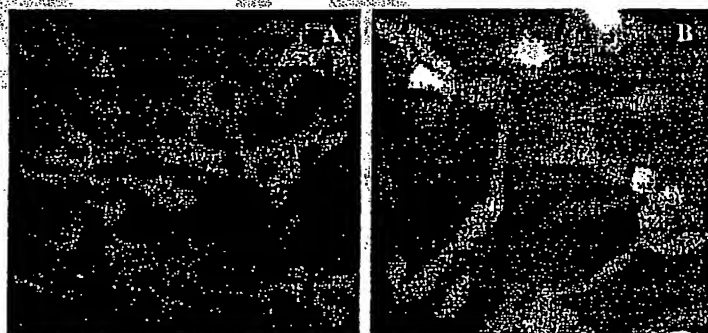
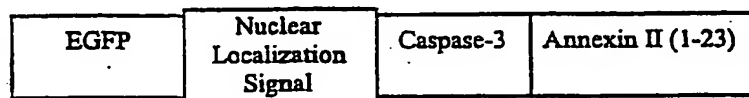
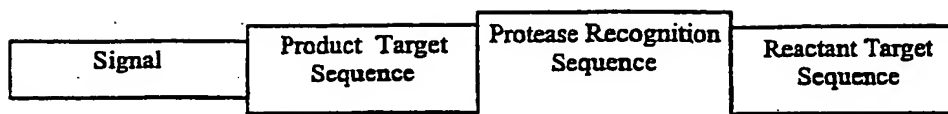


FIGURE 31



**Fig 3.** BHK cells transfected with DEVD-caspase biosensor. (A) Cells before stimulation of apoptosis. (B) Another field of cells after stimulation with 250  $\mu$ g/ml cis-platin (4 h).

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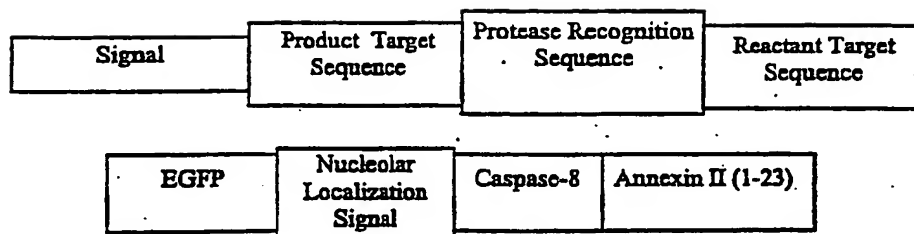


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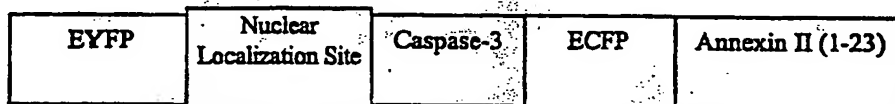
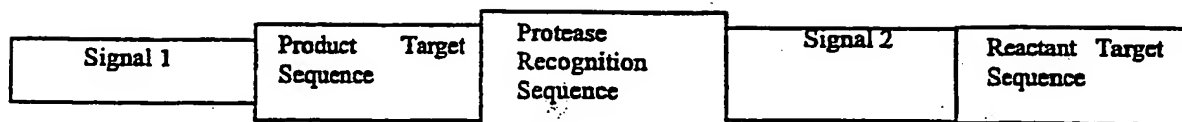
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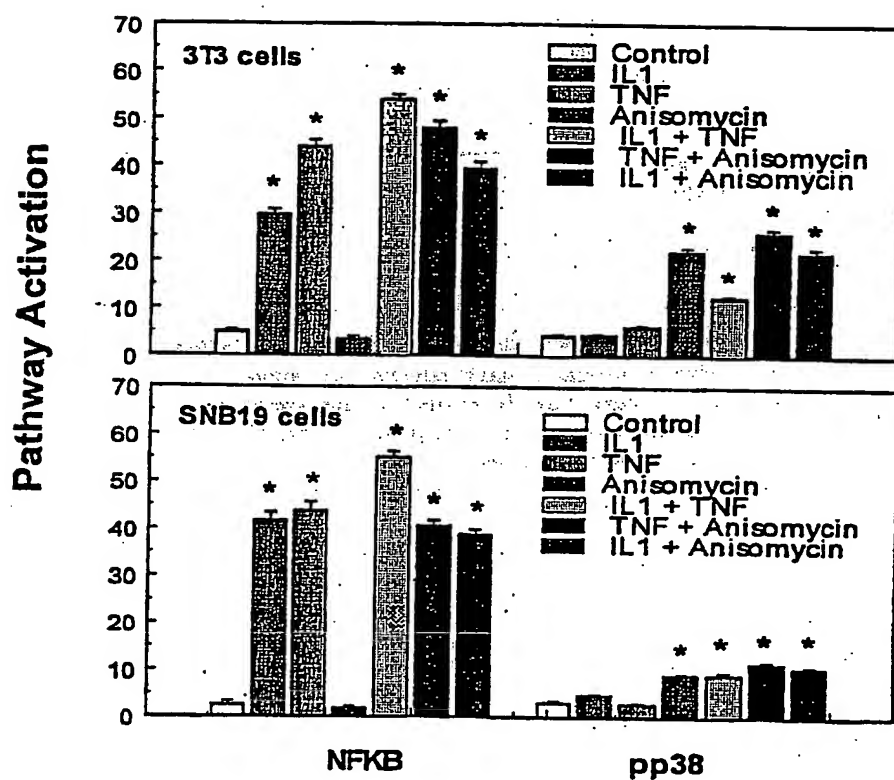
49/50



**Fig. 50.** Top: General design of biosensor with reactant and product containing separate targeting and signal sequences. Bottom: Specific example of this Approach—Caspase 3 biosensor with reactant targeted to cytoskeleton and product targeted to nucleus.

35

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**Fig. 36** Dual-labeling assay in two cell types with 3 drugs and 3 drug combinations. Treatments marked with an asterisk are different from controls at a 99% confidence level ( $p < 0.01$ ).

## SEQUENCE LISTING

<110> Giuliano, Kenneth A.  
Kapur, Ravi

<120> A System for Cell Based Screening

<130> 97-022-L

<140> To Be Assigned

<141> Filed Herewith

<160> 180

<170> PatentIn Ver. 2.0

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<222> (1)..(882)

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1				5				10					15			
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
			35				40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75					80		
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85					90					95			
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
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 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac 480  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160

ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc 528  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
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gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc 576  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
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ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg 624  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
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agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc 672  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
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gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc 720  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240

gga ctc aga tct ggc gcc ggc gct gga gcc gga gct ggc gcc gga gcc 768  
 Gly Leu Arg Ser Gly Ala Gly Ala Gly Ala Gly Ala Gly Ala  
 245 250 255

gac gag gtg gac ggc gcc ggc gcc gat gaa gta gat ggc gcc atg tct 816  
 Asp Glu Val Asp Gly Ala Gly Ala Asp Glu Val Asp Gly Ala Met Ser  
 260 265 270

act gtc cac gaa atc ctg tgc aag ctc agc ttg gag ggt gat cat tct 864  
 Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Asp His Ser  
 275 280 285

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 Thr Pro Pro Ser Ala Tyr  
 290

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accaccggca agctgcccggt gccctggccc accctcgtga ccaccctgac ctacggcgctg 1092

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 gccggagctg gcgccggagc cgacgaggtg gacggcgccg gcgccgatga agtagatggc 1692  
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 GFP-DEVD-Annexin II construct

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Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	35	40	45	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	50	55	60	
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	65	70	75	80
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	85	90	95	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	100	105	110	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	115	120	125	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	130	135	140	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	145	150	155	160
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	165	170	175	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	180	185	190	

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
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Gly Leu Arg Ser Gly Ala Gly Ala Gly Ala Gly Ala Gly Ala  
245 250 255

Asp Glu Val Asp Gly Ala Gly Ala Asp Glu Val Asp Gly Ala Met Ser  
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Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
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gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
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tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
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Phe	Gly	Tyr	Gly	Leu	Gln	Cys	Phe	Ala	Arg	Tyr	Pro	Asp	His	Met	Lys	
65				70				75				80				
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85				90					95				
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
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gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc tac cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag aag	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Lys	
225 230 235 240	
gga gac gaa gtg gac gga gcc gac ctc agt ctt gtg gat gcg ttg aca	768
Gly Asp Glu Val Asp Gly Ala Asp Leu Ser Leu Val Asp Ala Leu Thr	
245 250 255	
gaa cca cct cca gaa att gag gga gaa ata aag cga gac ttc atg gct	816
Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala	
260 265 270	
gcg ctg gag gca gag ccc tat gat gac atc gtg gga gaa act gtg gag	864
Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu	
275 280 285	
aaa act gag ttt att cct ctc ctg gat ggt gat gag aaa acc ggg aac	912
Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn	
290 295 300	
tca gag tcc aaa aag aaa ccc tgc tta gac act agc cag gtt gaa ggt	960
Ser Glu Ser Lys Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly	
305 310 315 320	
atc cca tct tct aaa cca aca ctc cta gcc aat ggt gat cat gga atg	1008
Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met	
325 330 335	
gag ggg aat aac act gca gga tct cca act gac --- -- --	1056
Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Asn	

340										345										350									
gtg	gac	tat	ccg	gat	tat	cag	agc	agc	cag	aac	tgg	cca	gaa	gat	gca														
Val	Asp	Tyr	Pro	Asp	Tyr	Gln	Ser	Ser	Gln	Asn	Trp	Pro	Glu	Asp	Ala														1104
		355							360						365														
agc	ttt	tgt	ttc	cag	cct	cag	caa	gtg	tta	gat	act	gac	cag	gct	gag														1152
Ser	Phe	Cys	Phe	Gln	Pro	Gln	Gln	Val	Leu	Asp	Thr	Asp	Gln	Ala	Glu														
		370							375						380														
ccc	ttt	aac	gag	cac	cgt	gat	gat	ggg	ttg	gca	gat	ctg	ctc	ttt	gtc														1200
Pro	Phe	Asn	Glu	His	Arg	Asp	Asp	Gly	Leu	Ala	Asp	Leu	Leu	Phe	Val														
		385							390						400														
tcc	agt	gga	ccc	acg	aac	gct	tct	gca	ttt	aca	gag	cga	gac	aat	cct														1248
Ser	Ser	Gly	Pro	Thr	Asn	Ala	Ser	Ala	Phe	Thr	Glu	Arg	Asp	Asn	Pro														
					405					410					415														
tca	gaa	gac	agt	tac	ggg	atg	ctt	ccc	tgt	gac	tca	ttt	gct	tcc	acg														1296
Ser	Glu	Asp	Ser	Tyr	Gly	Met	Leu	Pro	Cys	Asp	Ser	Phe	Ala	Ser	Thr														
					420					425					430														
gct	gtt	gta	tct	cag	gag	tgg	tct	gtg	gga	gcc	cca	aac	tct	cca	tgt														1344
Ala	Val	Val	Ser	Gln	Glu	Trp	Ser	Val	Gly	Ala	Pro	Asn	Ser	Pro	Cys														
					435					440					445														
tca	gag	tcc	tgt	gtc	tcc	cca	gag	gtt	act	ata	gaa	acc	cta	cag	cca														1392
Ser	Glu	Ser	Cys	Val	Ser	Pro	Glu	Val	Thr	Ile	Glu	Thr	Leu	Gln	Pro														
					450					455					460														
gca	aca	gag	ctc	tcc	aag	gca	gca	gaa	gtg	gaa	tca	gtg	aaa	gag	cag														1440
Ala	Thr	Glu	Leu	Ser	Lys	Ala	Ala	Glu	Val	Glu	Ser	Val	Lys	Glu	Gln														
					470					475					480														
ctg	cca	gct	aaa	gca	ttg	gaa	acg	atg	gca	gag	cag	acc	act	gat	gtg														1488
Leu	Pro	Ala	Lys	Ala	Leu	Glu	Thr	Met	Ala	Glu	Gln	Thr	Thr	Asp	Val														
					485					490					495														
gtg	cac	tct	cca	tcc	aca	gac	aca	aca	cca	ggc	cca	gac	aca	gag	gca														1536
Val	His	Ser	Pro	Ser	Thr	Asp	Thr	Thr	Pro	Gly	Pro	Asp	Thr	Glu	Ala														
					500					505					510														
gca	ctg	gct	aaa	gac	ata	gaa	gag	atc	acc	aag	cca	gat	gtg	ata	ttg														1584
Ala	Leu	Ala	Lys	Asp	Ile	Glu	Glu	Ile	Thr	Lys	Pro	Asp	Val	Ile	Leu														
					515					520					525														
gca	aat	gtc	acg	cag	cca	tct	act	gaa	tcg	gat	atg	ttc	ctg	gcc	cag														1632
Ala	Asn	Val	Thr	Gln	Pro	Ser	Thr	Glu	Ser	Asp	Met	Phe	Leu	Ala	Gln														
					530					535					540														
gac	atg	gaa	cta	ctc	aca	gga	aca	gag	gca	gcc	cac	gct	aac	aat	atc														1680
Asp	Met	Glu	Leu	Leu	Thr	Gly	Thr	Glu	Ala	Ala	His	Ala	Asn	Asn	Ile														
					550					555					560														
ata	ttg	cct	aca	gaa	cca	gac	gaa	tct	tca	acc	aag	gat	gta	gca	cca														1728
Ile	Leu	Pro	Thr	Glu	Pro	Asp	Glu	Ser	Ser	Thr	Lys	Asp	Val	Ala	Pro														
					565					570					575														
cct	atg	gaa	gaa	gaa	att	gtc	cca	ggc	aat	gat	acg	aca	tcc	ccc	aaa														1776
Pro	Met	Glu	Glu	Glu	Ile	Val	Pro	Gly	Asn	Asp	Thr	Thr	Ser	Pro	Lys														
					580					585					590														

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gaa aca gag aca aca ctt cca ata aaa atg gac ttg gca cca cct gag 1824
Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu
595 600 605

gat gtg tta ctt acc aaa gaa aca gaa cta gcc cca gcc aag ggc atg 1872
Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met
610 615 620

gtt tca ctc tca gaa ata gaa gag gct ctg gca aag aat gat gtt cgc 1920
Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg
625 630 635 640

tct gca gaa ata cct gtg gct cag gag aca gtg gtc tca gaa aca gag 1968
Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu
645 650 655

gtg gtc ctg gca aca gaa gtg gta ctg ccc tca gat ccc ata aca aca 2016
Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr
660 665 670

ttg aca aag gat gtg aca ctc ccc tta gaa gca gag aga ccg ttg gtg 2064
Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val
675 680 685

acg gac atg act cca tct ctg gaa aca gaa atg acc cta ggc aaa gag 2112
Thr Asp Met Thr Pro Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu
690 695 700

aca gct cca ccc aca gaa aca aat ttg ggc atg gcc aaa gac atg tct 2160
Thr Ala Pro Pro Thr Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser
705 710 715 720

cca ctc cca gaa tca gaa gtg act ctg ggc aag gac gtg gtt ata ctt 2208
Pro Leu Pro Glu Ser Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu
725 730 735

cca gaa aca aag gtg gct gag ttt aac aat gtg act cca ctt tca gaa 2256
Pro Glu Thr Lys Val Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu
740 745 750

gaa gag gta acc tca gtc aag gac atg tct ccg tct gca gaa aca gag 2304
Glu Glu Val Thr Ser Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu
755 760 765

gct ccc ctg gct aag aat gct gat ctg cac tca gga aca gag ctg att 2352
Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile
770 775 780

gtg gac aac agc atg gct cca gcc tcc gat ctt gca ctg ccc ttg gaa 2400
Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu
785 790 795 800

aca aaa gta gca aca gtt cca att aaa gac aaa gga tga 2439
Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly
805 810

```

<210> 4  
 <211> 812  
 <212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
EYFP-DEVD-MAPKDM construct

<400> 4

Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	1	5	10	15
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	20	25	30	
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	35	40	45	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	50	55	60	
Phe	Gly	Tyr	Gly	Leu	Gln	Cys	Phe	Ala	Arg	Tyr	Pro	Asp	His	Met	Lys	65	70	75	80
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	85	90	95	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	100	105	110	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	115	120	125	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	130	135	140	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	145	150	155	160
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	165	170	175	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	180	185	190	
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Tyr	Gln	Ser	Ala	Leu	195	200	205	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	210	215	220	
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Lys	225	230	235	240
Gly	Asp	Glu	Val	Asp	Gly	Ala	Asp	Leu	Ser	Leu	Val	Asp	Ala	Leu	Thr	245	250	255	
Glu	Pro	Pro	Pro	Glu	Ile	Glu	Gly	Glu	Ile	Lys	Arg	Asp	Phe	Met	Ala	260	265	270	
Ala	Leu	Glu	Ala	Glu	Pro	Tyr	Asp	Asp	Ile	Val	Gly	Glu	Thr	Val	Glu	275	280	285	

Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn  
 290 295 300  
 Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly  
 305 310 315 320  
 Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met  
 325 330 335  
 Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg.  
 340 345 350  
 Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala  
 355 360 365  
 Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu  
 370 375 380  
 Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val  
 385 390 395 400  
 Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro  
 405 410 415  
 Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr  
 420 425 430  
 Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys  
 435 440 445  
 Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro  
 450 455 460  
 Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu Gln  
 465 470 475 480  
 Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val  
 485 490 495  
 Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala  
 500 505 510  
 Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Pro Asp Val Ile Leu  
 515 520 525  
 Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln  
 530 535 540  
 Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile  
 545 550 555 560  
 Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala Pro  
 565 570 575  
 Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys  
 580 585 590  
 Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu  
 595 600 605  
 Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met

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        610                615                620
Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg
625                630                635                640
Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu
645                650                655
Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr
660                665                670
Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val
675                680                685
Thr Asp Met Thr Pro Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu
690                695                700
Thr Ala Pro Pro Thr Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser
705                710                715                720
Pro Leu Pro Glu Ser Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu
725                730                735
Pro Glu Thr Lys Val Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu
740                745                750
Glu Glu Val Thr Ser Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu
755                760                765
Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile
770                775                780
Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu
785                790                795                800
Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly
805                810

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<210> 5  
 <211> 2439  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1)..(2436)

<220>  
 <223> Description of Artificial Sequence:  
 EYFP-DEAD-MAPKDM construct

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<400> 5
atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg      48
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1                5                10                15

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc      96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
      20                25                30

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11

Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu	
275 280 285	
aaa act gag ttt att cct ctc ctg gat ggt gat gag aaa acc ggg aac	912
Lys Thr Glu Phe Ile Pro Leu Asp Gly Asp Glu Lys Thr Gly Asn	
290 295 300	
tca gag tcc aaa aag aaa ccc tgc tta gac act agc cag gtt gaa ggt	960
Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly	
305 310 315 320	
atc cca tct tct aaa cca aca ctc cta gcc aat ggt gat cat gga atg	1008
Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met	
325 330 335	
gag ggg aat aac act gca ggg tct cca act gac ttc ctt gaa gag aga	1056
Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg	
340 345 350	
gtg gac tat ccg gat tat cag agc agc cag aac tgg cca gaa gat gca	1104
Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala	
355 360 365	
agc ttt tgt ttc cag cct cag caa gtg tta gat act gac cag gct gag	1152
Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu	
370 375 380	
ccc ttt aac gag cac cgt gat gat ggt ttg gca gat ctg ctc ttt gtc	1200
Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val	
385 390 395 400	
tcc agt gga ccc acg aac gct tct gca ttt aca gag cga gac aat cct	1248
Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro	
405 410 415	
tca gaa gac agt tac ggt atg ctt ccc tgt gac tca ttt gct tcc acg	1296
Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr	
420 425 430	
gct gtt gta tct cag gag tgg tct gtg gga gcc cca aac tct cca tgt	1344
Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys	
435 440 445	
tca gag tcc tgt gtc tcc cca gag gtt act ata gaa acc cta cag cca	1392
Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro	
450 455 460	
gca aca gag ctc tcc aag gca gca gaa gtg gaa tca gtg aaa gag cag	1440
Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu Gln	
465 470 475 480	
ctg cca gct aaa gca ttg gaa acg atg gca gag cag acc act gat gtg	1488
Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val	
485 490 495	
gtg cac tct cca tcc aca gaa aca aca cca ggc cca gac aca gaa	1536
Val His Ser Pro Ser Thr Asp Thr Pro Gly Pro Asp Thr Glu Ala	
500 505 510	
gca ctg gct aaa gac ata gaa gag atc acc cca gat gtg ata ttg	1584
Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Asp Val Ile Leu	

515					520					525							
gca	aat	gtc	acg	cag	cca	tct	act	gaa	tcg	gat	atg	ttc	ctg	gcc	cag	1632	
Ala	Asn	Val	Thr	Gln	Pro	Ser	Thr	Glu	Ser	Asp	Met	Phe	Leu	Ala	Gln		
530					535					540							
gac	atg	gaa	cta	ctc	aca	gga	aca	gag	gca	gcc	cac	gct	aac	aat	atc	1680	
Asp	Met	Glu	Leu	Leu	Thr	Gly	Thr	Glu	Ala	Ala	His	Ala	Asn	Asn	Ile		
545					550					555					560		
ata	ttg	cct	aca	gaa	cca	gac	gaa	tct	tca	acc	aag	gat	gta	gca	cca	1728	
Ile	Leu	Pro	Thr	Glu	Pro	Asp	Glu	Ser	Ser	Thr	Lys	Asp	Val	Ala	Pro		
565					570					575							
cct	atg	gaa	gaa	gaa	att	gtc	cca	ggc	aat	gat	acg	aca	tcc	ccc	aaa	1776	
Pro	Met	Glu	Glu	Glu	Ile	Val	Pro	Gly	Asn	Asp	Thr	Thr	Ser	Pro	Lys		
580					585					590							
gaa	aca	gag	aca	aca	ctt	cca	ata	aaa	atg	gac	ttg	gca	cca	cct	gag	1824	
Glu	Thr	Glu	Thr	Thr	Leu	Pro	Ile	Lys	Met	Asp	Leu	Ala	Pro	Pro	Glu		
595					600					605							
gat	gtg	tta	ctt	acc	aaa	gaa	aca	gaa	cta	gcc	cca	gcc	aag	ggc	atg	1872	
Asp	Val	Leu	Leu	Thr	Lys	Glu	Thr	Glu	Leu	Ala	Pro	Ala	Lys	Gly	Met		
610					615					620							
gtt	tca	ctc	tca	gaa	ata	gaa	gag	gct	ctg	gca	aag	aat	gat	gtt	cgc	1920	
Val	Ser	Leu	Ser	Glu	Ile	Glu	Glu	Ala	Leu	Ala	Lys	Asn	Asp	Val	Arg		
625					630					635					640		
tct	gca	gaa	ata	cct	gtg	gct	cag	gag	aca	gtg	gtc	tca	gaa	aca	gag	1968	
Ser	Ala	Glu	Ile	Pro	Val	Ala	Gln	Glu	Thr	Val	Val	Ser	Glu	Thr	Glu		
645					650					655							
gtg	gtc	ctg	gca	aca	gaa	gtg	gta	ctg	ccc	tca	gat	ccc	ata	aca	aca	2016	
Val	Val	Leu	Ala	Thr	Glu	Val	Val	Leu	Pro	Ser	Asp	Pro	Ile	Thr	Thr		
660					665					670							
ttg	aca	aag	gat	gtg	aca	ctc	ccc	tta	gaa	gca	gag	aga	ccg	ttg	gtg	2064	
Leu	Thr	Lys	Asp	Val	Thr	Leu	Pro	Leu	Glu	Ala	Glu	Arg	Pro	Leu	Val		
675					680					685							
acg	gac	atg	act	cca	tct	ctg	gaa	aca	gaa	atg	acc	cta	ggc	aaa	gag	2112	
Thr	Asp	Met	Thr	Pro	Ser	Leu	Glu	Thr	Glu	Met	Thr	Leu	Gly	Lys	Glu		
690					695					700							
aca	gct	cca	ccc	aca	gaa	aca	aat	ttg	ggc	atg	gcc	aaa	gac	atg	tct	2160	
Thr	Ala	Pro	Pro	Thr	Glu	Thr	Asn	Leu	Gly	Met	Ala	Lys	Asp	Met	Ser		
705					710					715					720		
cca	ctc	cca	gaa	tca	gaa	gtg	act	ctg	ggc	aag	gac	gtg	gtt	ata	ctt	2208	
Pro	Leu	Pro	Glu	Ser	Glu	Val	Thr	Leu	Gly	Lys	Asp	Val	Val	Ile	Leu		
725					730					735							
cca	gaa	aca	aag	gtg	gct	gag	ttt	aac	aat	gtg	act	cca	ctt	tca	gaa	2256	
Pro	Glu	Thr	Lys	Val	Ala	Glu	Phe	Asn	Asn	Val	Thr	Pro	Leu	Ser	Glu		
740					745					750							
gaa	gag	gta	acc	tca	gtc	aag	gac	atg	tct	ccg	tct	gca	gaa	aca	gag	2304	
Glu	Glu	Val	Thr	Ser	Val	Lys	Asp	Met	Ser	Pro	Ser	Ala	Glu	Thr	Glu		
755					760					765							

gct ccc ctg gct aag aat gct gat ctg cac tca gga aca gag ctg att 2352  
 Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile  
 770 775 780

gtg gac aac agc atg gct cca gcc tcc gat ctt gca ctg ccc ttg gaa 2400  
 Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu  
 785 790 795 800

aca aaa gta gca aca gtt cca att aaa gac aaa gga tga 2439  
 Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly  
 805 810

<210> 6

<211> 812

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

EYFP-DEAD-MAPKDM construct

<400> 6

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu

195					200					205					
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe
210						215					220				
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Pro
225					230					235					240
Arg	Asp	Glu	Ala	Asp	Ser	Ala	Asp	Leu	Ser	Leu	Val	Asp	Ala	Leu	Thr
				245					250					255	
Glu	Pro	Pro	Pro	Glu	Ile	Glu	Gly	Glu	Ile	Lys	Arg	Asp	Phe	Met	Ala
			260					265					270		
Ala	Leu	Glu	Ala	Glu	Pro	Tyr	Asp	Asp	Ile	Val	Gly	Glu	Thr	Val	Glu
		275					280					285			
Lys	Thr	Glu	Phe	Ile	Pro	Leu	Leu	Asp	Gly	Asp	Glu	Lys	Thr	Gly	Asn
	290					295					300				
Ser	Glu	Ser	Lys	Lys	Lys	Pro	Cys	Leu	Asp	Thr	Ser	Gln	Val	Glu	Gly
305					310					315					320
Ile	Pro	Ser	Ser	Lys	Pro	Thr	Leu	Leu	Ala	Asn	Gly	Asp	His	Gly	Met
				325					330					335	
Glu	Gly	Asn	Asn	Thr	Ala	Gly	Ser	Pro	Thr	Asp	Phe	Leu	Glu	Glu	Arg
			340					345					350		
Val	Asp	Tyr	Pro	Asp	Tyr	Gln	Ser	Ser	Gln	Asn	Trp	Pro	Glu	Asp	Ala
	355						360					365			
Ser	Phe	Cys	Phe	Gln	Pro	Gln	Gln	Val	Leu	Asp	Thr	Asp	Gln	Ala	Glu
	370					375					380				
Pro	Phe	Asn	Glu	His	Arg	Asp	Asp	Gly	Leu	Ala	Asp	Leu	Leu	Phe	Val
385					390					395					400
Ser	Ser	Gly	Pro	Thr	Asn	Ala	Ser	Ala	Phe	Thr	Glu	Arg	Asp	Asn	Pro
				405					410					415	
Ser	Glu	Asp	Ser	Tyr	Gly	Met	Leu	Pro	Cys	Asp	Ser	Phe	Ala	Ser	Thr
			420					425					430		
Ala	Val	Val	Ser	Gln	Glu	Trp	Ser	Val	Gly	Ala	Pro	Asn	Ser	Pro	Cys
		435					440					445			
Ser	Glu	Ser	Cys	Val	Ser	Pro	Glu	Val	Thr	Ile	Glu	Thr	Leu	Gln	Pro
	450					455					460				
Ala	Thr	Glu	Leu	Ser	Lys	Ala	Ala	Glu	Val	Glu	Ser	Val	Lys	Glu	Gln
465					470					475					480
Leu	Pro	Ala	Lys	Ala	Leu	Glu	Thr	Met	Ala	Glu	Gln	Thr	Thr	Asp	Val
				485					490					495	
Val	His	Ser	Pro	Ser	Thr	Asp	Thr	Thr	Pro	Gly	Pro	Asp	Thr	Glu	Ala
			500					505					510		
Ala	Leu	Ala	Lys	Asp	Ile	Glu	Glu	Ile	Thr	Lys	Pro	Asp	Val	Ile	Leu
		515					520					525			

Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln  
 530 535 540  
 Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile  
 545 550 555 560  
 Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala Pro  
 565 570 575  
 Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys  
 580 585 590  
 Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu  
 595 600 605  
 Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met  
 610 615 620  
 Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg  
 625 630 635 640  
 Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu  
 645 650 655  
 Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr  
 660 665 670  
 Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val  
 675 680 685  
 Thr Asp Met Thr Pro Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu  
 690 695 700  
 Thr Ala Pro Pro Thr Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser  
 705 710 715 720  
 Pro Leu Pro Glu Ser Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu  
 725 730 735  
 Pro Glu Thr Lys Val Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu  
 740 745 750  
 Glu Glu Val Thr Ser Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu  
 755 760 765  
 Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile  
 770 775 780  
 Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu  
 785 790 795 800  
 Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly  
 805 810

&lt;210&gt; 7

&lt;211&gt; 864

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

<220>  
 <221> CDS  
 <222> (1)..(861)

<220>  
 <223> Description of Artificial Sequence: F25-MEK1  
 construct

<400> 7  
 atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48  
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15  
 gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60  
 ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240  
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288  
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa 336  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt 384  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac 432  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat 480  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160  
 gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528  
 Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190  
 cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt 624  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205

tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt 672  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac acc 720  
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Thr  
 225 230 235 240  
 ggt atg ccc aag aag aag ccg acg ccc atc cag ctg aac ccg gcc ccc 768  
 Gly Met Pro Lys Lys Lys Pro Thr Pro Ile Gln Leu Asn Pro Ala Pro  
 245 250 255  
 gac ggc tct gca gtt aac ggg acc agc tct gcg gag acc aac ttg gag 816  
 Asp Gly Ser Ala Val Asn Gly Thr Ser Ser Ala Glu Thr Asn Leu Glu  
 260 265 270  
 gcc ttg cag aag aag ctg gag gag cta gag ctt gat gag cag cag tga 864  
 Ala Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp Glu Gln Gln  
 275 280 285

&lt;210&gt; 8

&lt;211&gt; 287

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: F25-MEK1  
construct

&lt;400&gt; 8

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60  
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160



Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
                             165                            170                            175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
                             180                            185                            190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
                             195                            200                            205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
                             210                            215                            220  
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Thr  
                             225                            230                            235                            240  
 Gly Met Pro Lys Lys Lys Pro Thr Pro Ile Gln Leu Asn Pro Ala Pro  
                             245                            250                            255  
 Asp Gly Ser Ala Val Asn Gly Thr Ser Ser Ala Glu Thr Asn Leu Glu  
                             260                            265                            270  
 Ala Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp Glu Gln Gln  
                             275                            280                            285

<210> 9

<211> 876

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(873)

<220>

<223> Description of Artificial Sequence: F25-MEK2  
construct

<400> 9

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt	48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1                            5                            10                            15	
ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20                            25                            30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35                            40                            45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50                            55                            60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65                            70                            75                            80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	

	85	90	95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa				336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu				
	100	105	110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt				384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly				
	115	120	125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac				432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr				
	130	135	140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat				480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn				
	145	150	155	160
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc				528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser				
	165	170	175	
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc				576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly				
	180	185	190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt				624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu				
	195	200	205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt				672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe				
	210	215	220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac acc				720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Thr				
	225	230	235	240
ggc atg ctg gcc cgg agg aag ccg gtg ctg ccg gcg ctc acc atc aac				768
Gly Met Leu Ala Arg Arg Lys Pro Val Leu Pro Ala Leu Thr Ile Asn				
	245	250	255	
cct acc atc gcc gag ggc cca tcc cct acc agc gag ggc gcc tcc gag				816
Pro Thr Ile Ala Glu Gly Pro Ser Pro Thr Ser Glu Gly Ala Ser Glu				
	260	265	270	
gca aac ctg gtg gac ctg cag aag aag ctg gag gag ctg gaa ctt gac				864
Ala Asn Leu Val Asp Leu Gln Lys Lys Leu Glu Glu Glu Glu Leu Asp				
	275	280	285	
gag cag cag taa				876
Glu Gln Gln				
	290			

&lt;210&gt; 10

&lt;211&gt; 291

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: F25-MEK2  
construct

&lt;400&gt; 10

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60  
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160  
 Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Thr  
 225 230 235 240  
 Gly Met Leu Ala Arg Arg Lys Pro Val Leu Pro Ala Leu Thr Ile Asn  
 245 250 255  
 Pro Thr Ile Ala Glu Gly Pro Ser Pro Thr Ser Glu Gly Ala Ser Glu  
 260 265 270  
 Ala Asn Leu Val Asp Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp  
 275 280 285  
 Glu Gln Gln  
 290

<210> 11  
 <211> 889  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1)..(888)

<220>  
 <223> Description of Artificial Sequence: Caspase  
 3-DEVD-substrate construct

<400> 11  
 atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48  
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15  
 gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60  
 ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240  
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288  
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa 336  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt 384  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac 432  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat 480  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160  
 gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528  
 Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly

180	185	190	
cct gtc ctt tta cca gac aac cat	tac ctg tcc aca caa tct gcc ctt	624	
Pro Val Leu Leu Pro Asp Asn His	Tyr Leu Ser Thr Gln Ser Ala Leu		
195	200	205	
tcg aaa gat ccc aac gaa aag aga	gac cac atg gtc ctt ctt gag ttt	672	
Ser Lys Asp Pro Asn Glu Lys Arg	Asp His Met Val Leu Leu Glu Phe		
210	215	220	
gta aca gct gct ggg att aca cat	ggc atg gat gaa ctg tac aac tcc	720	
Val Thr Ala Ala Gly Ile Thr His	Gly Met Asp Glu Leu Tyr Asn Ser		
225	230	235	
gga aga agg aaa cga caa aag cga	tcg gct gtt aaa tct gaa gga aag	768	
Gly Arg Arg Lys Arg Gln Lys Arg	Ser Ala Val Lys Ser Glu Gly Lys		
245	250	255	
aga aag tgt gac gaa gtt gat gga	att gat gaa gta gca agt act atg	816	
Arg Lys Cys Asp Glu Val Asp Gly	Ile Asp Glu Val Ala Ser Thr Met		
260	265	270	
tct act gtc cac gaa atc ctg tgc	aag ctc agc ttg gag ggt gtt cat	864	
Ser Thr Val His Glu Ile Leu Cys	Lys Leu Ser Leu Glu Gly Val His		
275	280	285	
tct aca ccc cca agt acc cgg atc c		889	
Ser Thr Pro Pro Ser Thr Arg Ile			
290	295		

&lt;210&gt; 12

&lt;211&gt; 296

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Caspase  
3-DEVD-substrate construct

&lt;400&gt; 12

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220

Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser  
 225 230 235 240

Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Val Lys Ser Glu Gly Lys  
 245 250 255

Arg Lys Cys Asp Glu Val Asp Gly Ile Asp Glu Val Ala Ser Thr Met  
 260 265 270

Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val His  
 275 280 285

Ser Thr Pro Pro Ser Thr Arg Ile  
 290 295

&lt;210&gt; 13

&lt;211&gt; 846

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(846)

&lt;220&gt;

<223> Description of Artificial Sequence: Caspase  
 6-VEID-substrate construct

&lt;400&gt; 13

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48  
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc	720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser	
225 230 235 240	
gga aga agg aaa cga caa aag cga tcg aca aga ctt gtt gaa att gac	768
Gly Arg Arg Lys Arg Gln Lys Arg Ser Thr Arg Leu Val Glu Ile Asp	
245 250 255	
aac agt act atg agc aca gta cac gaa att tta tgt aaa tta agc tta	816
Asn Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu	
260 265 270	
gaa gga gta cac agt aca cca cca agc gca	846
Glu Gly Val His Ser Thr Pro Pro Ser Ala	

275

280

<210> 14  
 <211> 282  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Caspase  
 6-VEID-substrate construct

&lt;400&gt; 14

Met	Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	1	5	10	15
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	20	25	30	
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	35	40	45	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	50	55	60	
Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	65	70	75	80
Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	85	90	95	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	100	105	110	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	115	120	125	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	130	135	140	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	145	150	155	160
Gly	Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	165	170	175	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	180	185	190	
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	195	200	205	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	210	215	220	
Val	Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Asn	Ser	225	230	235	240
Gly	Arg	Arg	Lys	Arg	Gln	Lys	Arg	Ser	Thr	Arg	Leu	Val	Glu	Ile	Asp	245	250	255	



Asn Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu  
 260 265 270

Glu Gly Val His Ser Thr Pro Pro Ser Ala  
 275 280

<210> 15

<211> 876

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(876)

<220>

<223> Description of Artificial Sequence: Caspase 8-VETD  
 construct

<400> 15

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt	48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	

gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528  
 Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175

gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190

cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt 624  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205

tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt 672  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220

gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc 720  
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser  
 225 230 235 240

gga aga agc aaa cga caa aag cga tcg tat gaa aaa gga ata cca gtt 768  
 Gly Arg Ser Lys Arg Gln Lys Arg Ser Tyr Glu Lys Gly Ile Pro Val  
 245 250 255

gaa aca gac agc gaa gag caa gct tat agt act atg tct act gtc cac 816  
 Glu Thr Asp Ser Glu Glu Gln Ala Tyr Ser Thr Met Ser Thr Val His  
 260 265 270

gaa atc ctg tgc aag ctc agc ttg gag ggt gtt cat tct aca ccc cca 864  
 Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val His Ser Thr Pro Pro  
 275 280 285

agt gcc gga tcc 876  
 Ser Ala Gly Ser  
 290

&lt;210&gt; 16

&lt;211&gt; 292

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Caspase 8-VETD  
construct

&lt;400&gt; 16

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
50 55 60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
65 70 75 80

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<210> 17
<211> 906:
<212> DNA
<213> Artificial Sequence
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<220>  
<223> Description of Artificial Sequence: Cas 3-multiple  
DEVD construct

29

gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc	720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser	
225 230 235 240	
gga aga agg aaa cga caa aag cga tcg gca ggt gac gaa gtt gat gca	768
Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Asp Glu Val Asp Ala	
245 250 255	

ggt gac gaa gtt gat gca ggt gac gaa gtt gat gca ggt gac gaa gtt 816  
 Gly Asp Glu Val Asp Ala Gly Asp Glu Val Asp Ala Gly Asp Glu Val  
                   260                  265                  270

gac gca ggt agt act atg tct act gtc cac gaa atc ctg tgc aag ctc 864  
 Asp Ala Gly Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu  
                   275                  280                  285

agc ttg gag ggt gtt cat tct aca ccc cca agt gcc gga tcc 906  
 Ser Leu Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser  
                   290                  295                  300

<210> 18

<211> 302

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Cas 3-multiple  
 DEVD construct

<400> 18

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
   1                  5                  10                  15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
                   20                  25                  30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
                   35                  40                  45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
                   50                  55                  60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
   65                  70                  75                  80

Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
                   85                  90                  95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
                   100                  105                  110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
                   115                  120                  125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
                   130                  135                  140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
   145                  150                  155                  160

Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
                   165                  170                  175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
                   180                  185                  190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
                   195                  200                  205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220

Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser  
 225 230 235 240

Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Asp Glu Val Asp Ala  
 245 250 255

Gly Asp Glu Val Asp Ala Gly Asp Glu Val Asp Ala Gly Asp Glu Val  
 260 265 270

Asp Ala Gly Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu  
 275 280 285

Ser Leu Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser  
 290 295 300

<210> 19  
 <211> 906  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1)..(885)

<220>  
 <223> Description of Artificial Sequence: Caspase  
 8-multiple VETD construct

<400> 19  
 atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48  
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240  
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288  
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa 336  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

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gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt 384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115              120              125

att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac 432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130              135              140

aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat 480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
      145              150              155              160

gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
              165              170              175

gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
              180              185              190

cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt 624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
              195              200              205

tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt 672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
      210              215              220

gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc 720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser
      225              230              235              240

gga aga agg aaa cga caa aag cga tcg gca ggt gtt gaa aca gac gca 768
Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Val Glu Thr Asp Ala
              245              250              255

ggg gtt gaa aca gac gca ggt gtt gaa aca gac gca ggt gtt gaa aca 816
Gly Val Glu Thr Asp Ala Gly Val Glu Thr Asp Ala Gly Val Glu Thr
              260              265              270

gac gca ggt agt act atg tct act gtc cac gaa atc ctg tgc aag ctc 864
Asp Ala Gly Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu
      275              280              285

agc ttg gag ggt gtt cat tct acacccccaa gtgccggatc c 906
Ser Leu Glu Gly Val His Ser
      290              295

```

&lt;210&gt; 20

&lt;211&gt; 295

&lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Caspase  
8-multiple VETD construct

&lt;400&gt; 20

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu

1	5	10	15
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	20	25	30
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	35	40	45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	50	55	60
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	65	70	75
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	85	90	95
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	100	105	110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	115	120	125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	130	135	140
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	145	150	155
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	165	170	175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	180	185	190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	195	200	205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	210	215	220
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser	225	230	235
Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Val Glu Thr Asp Ala	245	250	255
Gly Val Glu Thr Asp Ala Gly Val Glu Thr Asp Ala Gly Val Glu Thr	260	265	270
Asp Ala Gly Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu	275	280	285
Ser Leu Glu Gly Val His Ser	290	295	

&lt;210&gt; 21

&lt;211&gt; 4833

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence



&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(4830)

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:

EYFP-DEVD-MAP4-EBFP construct

&lt;400&gt; 21

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5					10					15		
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25						30		
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
			35				40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ttc	ggc	tac	ggc	ctg	cag	tgc	ttc	gcc	cgc	tac	ccc	gac	cac	atg	aag	240
Phe	Gly	Tyr	Gly	Leu	Gln	Cys	Phe	Ala	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75					80		
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85					90						95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130					135					140					
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155				160		
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
			165					170						175		
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180					185					190			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	tac	cag	tcc	gcc	ctg	624
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Tyr	Gln	Ser	Ala	Leu	
		195					200					205				

agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	
210						215					220					
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag	aag	720
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Lys	
225					230					235					240	
gga	gac	gaa	gtg	gac	gga	atg	gcc	gac	ctc	agt	ctt	gtg	gat	gcg	ttg	768
Gly	Asp	Glu	Val	Asp	Gly	Met	Ala	Asp	Leu	Ser	Leu	Val	Asp	Ala	Leu	
				245					250					255		
aca	gaa	cca	cct	cca	gaa	att	gag	gga	gaa	ata	aag	cga	gac	ttc	atg	816
Thr	Glu	Pro	Pro	Pro	Glu	Ile	Glu	Gly	Glu	Ile	Lys	Arg	Asp	Phe	Met	
			260					265					270			
gct	gcg	ctg	gag	gca	gag	ccc	tat	gat	gac	atc	gtg	gga	gaa	act	gtg	864
Ala	Ala	Leu	Glu	Ala	Glu	Pro	Tyr	Asp	Asp	Ile	Val	Gly	Glu	Thr	Val	
		275					280					285				
gag	aaa	act	gag	ttt	att	cct	ctc	ctg	gat	ggg	gat	gag	aaa	acc	ggg	912
Glu	Lys	Thr	Glu	Phe	Ile	Pro	Leu	Leu	Asp	Gly	Asp	Glu	Lys	Thr	Gly	
	290					295					300					
aac	tca	gag	tcc	aaa	aag	aaa	ccc	tgc	tta	gac	act	agc	cag	gtt	gaa	960
Asn	Ser	Glu	Ser	Lys	Lys	Lys	Pro	Cys	Leu	Asp	Thr	Ser	Gln	Val	Glu	
305					310					315					320	
ggg	atc	cca	tct	tct	aaa	cca	aca	ctc	cta	gcc	aat	ggg	gat	cat	gga	1008
Gly	Ile	Pro	Ser	Ser	Lys	Pro	Thr	Leu	Leu	Ala	Asn	Gly	Asp	His	Gly	
				325					330					335		
atg	gag	ggg	aat	aac	act	gca	ggg	tct	cca	act	gac	ttc	ctt	gaa	gag	1056
Met	Glu	Gly	Asn	Asn	Thr	Ala	Gly	Ser	Pro	Thr	Asp	Phe	Leu	Glu	Glu	
			340					345					350			
aga	gtg	gac	tat	ccg	gat	tat	cag	agc	agc	cag	aac	tgg	cca	gaa	gat	1104
Arg	Val	Asp	Tyr	Pro	Asp	Tyr	Gln	Ser	Ser	Gln	Asn	Trp	Pro	Glu	Asp	
		355					360					365				
gca	agc	ttt	tgt	ttc	cag	cct	cag	caa	gtg	tta	gat	act	gac	cag	gct	1152
Ala	Ser	Phe	Cys	Phe	Gln	Pro	Gln	Gln	Val	Leu	Asp	Thr	Asp	Gln	Ala	
		370				375					380					
gag	ccc	ttt	aac	gag	cac	cgt	gat	gat	ggg	ttg	gca	gat	ctg	ctc	ttt	1200
Glu	Pro	Phe	Asn	Glu	His	Arg	Asp	Asp	Gly	Leu	Ala	Asp	Leu	Leu	Phe	
	385				390					395					400	
gtc	tcc	agt	gga	ccc	acg	aac	gct	tct	gca	ttt	aca	gag	cga	gac	aat	1248
Val	Ser	Ser	Gly	Pro	Thr	Asn	Ala	Ser	Ala	Phe	Thr	Glu	Arg	Asp	Asn	
				405					410					415		
cct	tca	gaa	gac	agt	tac	ggg	atg	ctt	ccc	tgt	gac	tca	ttt	gct	tcc	1296
Pro	Ser	Glu	Asp	Ser	Tyr	Gly	Met	Leu	Pro	Cys	Asp	Ser	Phe	Ala	Ser	
			420					425					430			
acg	gct	gtt	gta	tct	cag	gag	tgg	tct	gtg	gga	gcc	cca	aac	tct	cca	1344
Thr	Ala	Val	Val	Ser	Gln	Glu	Trp	Ser	Val	Gly	Ala	Pro	Asn	Ser	Pro	
		435					440					445				

tgt tca gag tcc tgt gtc tcc cca gag gtt act ata gaa acc cta cag Cys Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln 450 455 460	1392
cca gca aca gag ctc tcc aag gca gca gaa gtg gaa tca gtg aaa gag Pro Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu 465 470 475 480	1440
cag ctg cca gct aaa gca ttg gaa acg atg gca gag cag acc act gat Gln Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp 485 490 495	1488
gtg gtg cac tct cca tcc aca gac aca aca cca ggc cca gac aca gag Val Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly Pro Thr Glu 500 505 510	1536
gca gca ctg gct aaa gac ata gaa gag atc acc aag cca gat gtg ata Ala Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Pro Asp Val Ile 515 520 525	1584
ttg gca aat gtc acg cag cca tct act gaa tcg gat atg ttc ctg gcc Leu Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala 530 535 540	1632
cag gac atg gaa cta ctc aca gga aca gag gca gcc cac gct aac aat Gln Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn 545 550 555 560	1680
atc ata ttg cct aca gaa cca gac gaa tct tca acc aag gat gta gca Ile Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala 565 570 575	1728
cca cct atg gaa gaa gaa att gtc cca ggc aat gat acg aca tcc ccc Pro Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro 580 585 590	1776
aaa gaa aca gag aca aca ctt cca ata aaa atg gac ttg gca cca cct Lys Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro 595 600 605	1824
gag gat gtg tta ctt acc aaa gaa aca gaa cta gcc cca gcc aag ggc Glu Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly 610 615 620	1872
atg gtt tca ctc tca gaa ata gaa gag gct ctg gca aag aat gat gtt Met Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val 625 630 635 640	1920
cgc tct gca gaa ata cct gtg gct cag gag aca gtg gtc tca gaa aca Arg Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr 645 650 655	1968
gag gtg gtc ctg gca aca gaa gtg gta ctg ccc tca gat ccc ata aca Glu Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr 660 665 670	2016
aca ttg aca aag gat gtg aca ctc ccc tta gaa gca gag aga ccg ttg Thr Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu 675 680 685	2064
gtg acg gac atg act cca tct ctg gaa aca gaa atg acc cta ggc aaa	2112

Val	Thr	Asp	Met	Thr	Pro	Ser	Leu	Glu	Thr	Glu	Met	Thr	Leu	Gly	Lys	
690						695					700					
gag	aca	gct	cca	ccc	aca	gaa	aca	aat	ttg	ggc	atg	gcc	aaa	gac	atg	2160
Glu	Thr	Ala	Pro	Pro	Thr	Glu	Thr	Asn	Leu	Gly	Met	Ala	Lys	Asp	Met	
705					710					715					720	
tct	cca	ctc	cca	gaa	tca	gaa	gtg	act	ctg	ggc	aag	gac	gtg	gtt	ata	2208
Ser	Pro	Leu	Pro		Ser	Glu	Val	Thr	Leu	Gly	Lys	Asp	Val	Val	Ile	
				725					730					735		
ctt	cca	gaa	aca	aag	gtg	gct	gag	ttt	aac	aat	gtg	act	cca	ctt	tca	2256
Leu	Pro	Glu	Thr	Lys	Val	Ala	Glu	Phe	Asn	Asn	Val	Thr	Pro	Leu	Ser	
			740					745						750		
gaa	gaa	gag	gta	acc	tca	gtc	aag	gac	atg	tct	ccg	tct	gca	gaa	aca	2304
Glu	Glu	Glu	Val	Thr	Ser	Val	Lys	Asp	Met	Ser	Pro	Ser	Ala	Glu	Thr	
			755				760					765				
gag	gct	ccc	ctg	gct	aag	aat	gct	gat	ctg	cac	tca	gga	aca	gag	ctg	2352
Glu	Ala	Pro	Leu	Ala	Lys	Asn	Ala	Asp	Leu	His	Ser	Gly	Thr	Glu	Leu	
770						775					780					
att	gtg	gac	aac	agc	atg	gct	cca	gcc	tcc	gat	ctt	gca	ctg	ccc	ttg	2400
Ile	Val	Asp	Asn	Ser	Met	Ala	Pro	Ala	Ser	Asp	Leu	Ala	Leu	Pro	Leu	
785					790					795					800	
gaa	aca	aaa	gta	gca	aca	gtt	cca	att	aaa	gac	aaa	gga	act	gta	cag	2448
Glu	Thr	Lys	Val	Ala	Thr	Val	Pro	Ile	Lys	Asp	Lys	Gly	Thr	Val	Gln	
				805					810					815		
act	gaa	gaa	aaa	cca	cgt	gaa	gac	tcc	cag	tta	gca	tct	atg	cag	cac	2496
Thr	Glu	Glu	Lys	Pro	Arg	Glu	Asp	Ser	Gln	Leu	Ala	Ser	Met	Gln	His	
			820					825						830		
aag	gga	cag	tca	aca	gta	cct	cct	tgc	acg	gct	tca	cca	gaa	cca	gtc	2544
Lys	Gly	Gln	Ser	Thr	Val	Pro	Pro	Cys	Thr	Ala	Ser	Pro	Glu	Pro	Val	
			835				840					845				
aaa	gct	gca	gaa	caa	atg	tct	acc	tta	cca	ata	gat	gca	cct	tct	cca	2592
Lys	Ala	Ala	Glu	Gln	Met	Ser	Thr	Leu	Pro	Ile	Asp	Ala	Pro	Ser	Pro	
			850			855					860					
tta	gag	aac	tta	gag	cag	aag	gaa	acg	cct	ggc	agc	cag	cct	tct	gag	2640
Leu	Glu	Asn	Leu	Glu	Gln	Lys	Glu	Thr	Pro	Gly	Ser	Gln	Pro	Ser	Glu	
865					870					875					880	
cct	tgc	tca	gga	gta	tcc	cgg	caa	gaa	gaa	gca	aag	gct	gct	gta	ggt	2688
Pro	Cys	Ser	Gly	Val	Ser	Arg	Gln	Glu	Glu	Ala	Lys	Ala	Ala	Val	Gly	
				885					890					895		
gtg	act	gga	aat	gac	atc	act	acc	ccg	cca	aac	aag	gag	cca	cca	cca	2736
Val	Thr	Gly	Asn	Asp	Ile	Thr	Thr	Pro	Pro	Asn	Lys	Glu	Pro	Pro	Pro	
			900					905						910		
agc	cca	gaa	aag	aaa	gca	aag	cct	ttg	gcc	acc	act	caa	cct	gca	aag	2784
Ser	Pro	Glu	Lys	Lys	Ala	Lys	Pro	Leu	Ala	Thr	Thr	Gln	Pro	Ala	Lys	
			915				920					925				
act	tca	aca	tcg	aaa	gcc	aaa	aca	cag	ccc	act	tct	ctc	cct	aag	caa	2832
Thr	Ser	Thr	Ser	Lys	Ala	Lys	Thr	Gln	Pro	Thr	Ser	Leu	Pro	Lys	Gln	

930	935	940	
cca gct ccc acc acc tct ggt ggg ttg aat aaa aaa ccc atg agc ctc			2880
Pro Ala Pro Thr Thr Ser Gly Gly Leu Asn Lys Lys Pro Met Ser Leu			
945	950	955	960
gcc tca ggc tca gtg cca gct gcc cca cac aaa cgc cct gct gct gcc			2928
Ala Ser Gly Ser Val Pro Ala Ala Pro His Lys Arg Pro Ala Ala Ala			
	965	970	975
act gct act gcc agg cct tcc acc cta cct gcc aga gac gtg aag cca			2976
Thr Ala Thr Ala Arg Pro Ser Thr Leu Pro Ala Arg Asp Val Lys Pro			
	980	985	990
aag cca att aca gaa gct aag gtt gcc gaa aag cgg acc tct cca tcc			3024
Lys Pro Ile Thr Glu Ala Lys Val Ala Glu Lys Arg Thr Ser Pro Ser			
	995	1000	1005
aag cct tca tct gcc cca gcc ctc aaa cct gga cct aaa acc acc cca			3072
Lys Pro Ser Ser Ala Pro Ala Leu Lys Pro Gly Pro Lys Thr Thr Pro			
	1010	1015	1020
acc gtt tca aaa gcc aca tct ccc tca act ctt gtt tcc act gga cca			3120
Thr Val Ser Lys Ala Thr Ser Pro Ser Thr Leu Val Ser Thr Gly Pro			
	1025	1030	1035
agt agt aga agt cca gct aca act ctg cct aag agg cca acc agc atc			3168
Ser Ser Arg Ser Pro Ala Thr Thr Leu Pro Lys Arg Pro Thr Ser Ile			
	1045	1050	1055
aag act gag ggg aaa cct gct gat gtc aaa agg atg act gct aag tct			3216
Lys Thr Glu Gly Lys Pro Ala Asp Val Lys Arg Met Thr Ala Lys Ser			
	1060	1065	1070
gcc tca gct gac ttg agt cgc tca aag acc acc tct gcc agt tct gtg			3264
Ala Ser Ala Asp Leu Ser Arg Ser Lys Thr Thr Ser Ala Ser Ser Val			
	1075	1080	1085
aag aga aac acc act ccc act ggg gca gca ccc cca gca ggg atg act			3312
Lys Arg Asn Thr Thr Pro Thr Gly Ala Ala Pro Pro Ala Gly Met Thr			
	1090	1095	1100
tcc act cga gtc aag ccc atg tct gca cct agc cgc tct tct ggg gct			3360
Ser Thr Arg Val Lys Pro Met Ser Ala Pro Ser Arg Ser Ser Gly Ala			
	1105	1110	1115
ctt tct gtg gac aag aag ccc act tcc act aag cct agc tcc tct gct			3408
Leu Ser Val Asp Lys Lys Pro Thr Ser Thr Lys Pro Ser Ser Ser Ala			
	1125	1130	1135
ccc agg gtg agc cgc ctg gcc aca act gtt tct gcc cct gac ctg aag			3456
Pro Arg Val Ser Arg Leu Ala Thr Thr Val Ser Ala Pro Asp Leu Lys			
	1140	1145	1150
agt gtt cgc tcc aag gtc ggc tct aca gaa aac atc aaa cac cag cct			3504
Ser Val Arg Ser Lys Val Gly Ser Thr Glu Asn Ile Lys His Gln Pro			
	1155	1160	1165
gga gga ggc cgg gcc aaa gtc aag aaa aaa aag gca cct acc acc			3552
Gly Gly Gly Arg Ala Lys Val Glu Lys Lys Thr Glu Ala Ala Thr Thr			
	1170	1175	1180

gct ggg aag cct gaa cct aat gca gtc act aaa gca gcc ggc tcc att Ala Gly Lys Pro Glu Pro Asn Ala Val Thr Lys Ala Ala Gly Ser Ile 1185 1190 1195 1200	3600
gcg agt gca cag aaa ccg cct gct ggg aaa gtc cag ata gta tcc aaa Ala Ser Ala Gln Lys Pro Pro Ala Gly Lys Val Gln Ile Val Ser Lys 1205 1210 1215	3648
aaa gtg agc tac agt cat att caa tcc aag tgt gtt tcc aag gac aat Lys Val Ser Tyr Ser His Ile Gln Ser Lys Cys Val Ser Lys Asp Asn 1220 1225 1230	3696
att aag cat gtc cct gga tgt ggc aat gtt cag att cag aac aag aaa Ile Lys His Val Pro Gly Cys Gly Asn Val Gln Ile Gln Asn Lys Lys 1235 1240 1245	3744
gtg gac ata tcc aag gtc tcc tcc aag tgt ggg tcc aaa gct aat atc Val Asp Ile Ser Lys Val Ser Ser Lys Cys Gly Ser Lys Ala Asn Ile 1250 1255 1260	3792
aag cac aag cct ggt gga gga gat gtc aag att gaa agt cag aag ttg Lys His Lys Pro Gly Gly Gly Asp Val Lys Ile Glu Ser Gln Lys Leu 1265 1270 1275 1280	3840
aac ttc aag gag aag gcc caa gcc aaa gtg gga tcc ctt gat aac gtt Asn Phe Lys Glu Lys Ala Gln Ala Lys Val Gly Ser Leu Asp Asn Val 1285 1290 1295	3888
ggc cac ttt cct gca gga ggt gcc gtg aag act gag ggc ggt ggc agt Gly His Phe Pro Ala Gly Gly Ala Val Lys Thr Glu Gly Gly Gly Ser 1300 1305 1310	3936
gag gcc ctt ccg tgt cca ggc ccc ccc gct ggg gag gag cca gtc atc Glu Ala Leu Pro Cys Pro Gly Pro Pro Ala Gly Glu Glu Pro Val Ile 1315 1320 1325	3984
cct gag gct gcg cct gac cgt ggc gcc cct act tca gcc agt ggc ctc Pro Glu Ala Ala Pro Asp Arg Gly Ala Pro Thr Ser Ala Ser Gly Leu 1330 1335 1340	4032
agt ggc cac acc acc ctg tca ggg ggt ggt gac caa agg gag ccc cag Ser Gly His Thr Thr Leu Ser Gly Gly Gly Asp Gln Arg Glu Pro Gln 1345 1350 1355 1360	4080
acc ttg gac agc cag atc cag gag aca agc atc atg gtg agc aag ggc Thr Leu Asp Ser Gln Ile Gln Glu Thr Ser Ile Met Val Ser Lys Gly 1365 1370 1375	4128
gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg gac ggc Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly 1380 1385 1390	4176
gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag ggc gat Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp 1395 1400 1405	4224
gcc acc tac ggc aag ctg acc ctg aag ttc atc tgc acc acc ggc aag Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys 1410 1415 1420	4272

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ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc ctg acc cac ggc gtg 4320
Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr His Gly Val
1425 1430 1435 1440

cag tgc ttc agc cgc tac ccc gac cac atg aag cag cac gac ttc ttc 4368
Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe
1445 1450 1455

aag tcc gcc atg ccc gaa ggc tac gtc cag gag cgc acc atc ttc ttc 4416
Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe
1460 1465 1470

aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc gag ggc 4464
Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly
1475 1480 1485

gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag 4512
Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu
1490 1495 1500

gac ggc aac atc ctg ggg cac aag ctg gag tac aac ttc aac agc cac 4560
Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Phe Asn Ser His
1505 1510 1515 1520

aac gtc tat atc atg gcc gac aag cag aag aac ggc atc aag gtg aac 4608
Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn
1525 1530 1535

ttc aag atc cgc cac aac atc gag gac ggc agc gtg cag ctc gcc gac 4656
Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp
1540 1545 1550

cac tac cag cag aac acc ccc atc ggc gac ggc ccc gtg ctg ctg ccc 4704
His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro
1555 1560 1565

gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa gac ccc aac 4752
Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn
1570 1575 1580

gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggc 4800
Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly
1585 1590 1595 1600

atc act ctc ggc atg gac gag ctg tac aag tag 4833
Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
1605 1610

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&lt;210&gt; 22

&lt;211&gt; 1610

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:  
EYFP-DEVD-MAP4-EBFP construct

&lt;400&gt; 22

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15

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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60  
 Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Lys  
 225 230 235 240  
 Gly Asp Glu Val Asp Gly Met Ala Asp Leu Ser Leu Val Asp Ala Leu  
 245 250 255  
 Thr Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys Arg Asp Phe Met  
 260 265 270  
 Ala Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val Gly Glu Thr Val  
 275 280 285  
 Glu Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly  
 290 295 300  
 Asn Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr Ser Gln Val Glu  
 305 310 315 320  
 Gly Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn Gly Asp His Gly  
 325 330 335



Met Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu  
 340 345 350  
 Arg Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp  
 355 360 365  
 Ala Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp Thr Asp Gln Ala  
 370 375 380  
 Glu Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe  
 385 390 395 400  
 Val Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn  
 405 410 415  
 Pro Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp Ser Phe Ala Ser  
 420 425 430  
 Thr Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala Pro Asn Ser Pro  
 435 440 445  
 Cys Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln  
 450 455 460  
 Pro Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu  
 465 470 475 480  
 Gln Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp  
 485 490 495  
 Val Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu  
 500 505 510  
 Ala Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Pro Asp Val Ile  
 515 520 525  
 Leu Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala  
 530 535 540  
 Gln Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn  
 545 550 555 560  
 Ile Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala  
 565 570 575  
 Pro Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro  
 580 585 590  
 Lys Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro  
 595 600 605  
 Glu Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly  
 610 615 620  
 Met Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val  
 625 630 635 640  
 Arg Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr  
 645 650 655  
 Glu Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr

660	665	670
Thr Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu 675 680 685		
Val Thr Asp Met Thr Pro Ser Leu Glu Thr Glu Met Thr Leu Gly Lys 690 695 700		
Glu Thr Ala Pro Pro Thr Glu Thr Asn Leu Gly Met Ala Lys Asp Met 705 710 715 720		
Ser Pro Leu Pro Glu Ser Glu Val Thr Leu Gly Lys Asp Val Val Ile 725 730 735		
Leu Pro Glu Thr Lys Val Ala Glu Phe Asn Asn Val Thr Pro Leu Ser 740 745 750		
Glu Glu Glu Val Thr Ser Val Lys Asp Met Ser Pro Ser Ala Glu Thr 755 760 765		
Glu Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu 770 775 780		
Ile Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu 785 790 795 800		
Glu Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly Thr Val Gln 805 810 815		
Thr Glu Glu Lys Pro Arg Glu Asp Ser Gln Leu Ala Ser Met Gln His 820 825 830		
Lys Gly Gln Ser Thr Val Pro Pro Cys Thr Ala Ser Pro Glu Pro Val 835 840 845		
Lys Ala Ala Glu Gln Met Ser Thr Leu Pro Ile Asp Ala Pro Ser Pro 850 855 860		
Leu Glu Asn Leu Glu Gln Lys Glu Thr Pro Gly Ser Gln Pro Ser Glu 865 870 875 880		
Pro Cys Ser Gly Val Ser Arg Gln Glu Glu Ala Lys Ala Ala Val Gly 885 890 895		
Val Thr Gly Asn Asp Ile Thr Thr Pro Pro Asn Lys Glu Pro Pro Pro 900 905 910		
Ser Pro Glu Lys Lys Ala Lys Pro Leu Ala Thr Thr Gln Pro Ala Lys 915 920 925		
Thr Ser Thr Ser Lys Ala Lys Thr Gln Pro Thr Ser Leu Pro Lys Gln 930 935 940		
Pro Ala Pro Thr Thr Ser Gly Gly Leu Asn Lys Lys Pro Met Ser Leu 945 950 955 960		
Ala Ser Gly Ser Val Pro Ala Ala Pro His Lys Arg Pro Ala Ala Ala 965 970 975		
Thr Ala Thr Ala Arg Pro Ser Thr Leu Pro Ala Arg Asp Val Lys Pro 980 985 990		

Lys Pro Ile Thr Glu Ala Lys Val Ala Glu Lys Arg Thr Ser Pro Ser  
 995 1000 1005  
 Lys Pro Ser Ser Ala Pro Ala Leu Lys Pro Gly Pro Lys Thr Thr Pro  
 1010 1015 1020  
 Thr Val Ser Lys Ala Thr Ser Pro Ser Thr Leu Val Ser Thr Gly Pro  
 1025 1030 1035 1040  
 Ser Ser Arg Ser Pro Ala Thr Thr Leu Pro Lys Arg Pro Thr Ser Ile  
 1045 1050 1055  
 Lys Thr Glu Gly Lys Pro Ala Asp Val Lys Arg Met Thr Ala Lys Ser  
 1060 1065 1070  
 Ala Ser Ala Asp Leu Ser Arg Ser Lys Thr Thr Ser Ala Ser Ser Val  
 1075 1080 1085  
 Lys Arg Asn Thr Thr Pro Thr Gly Ala Ala Pro Pro Ala Gly Met Thr  
 1090 1095 1100  
 Ser Thr Arg Val Lys Pro Met Ser Ala Pro Ser Arg Ser Ser Gly Ala  
 1105 1110 1115 1120  
 Leu Ser Val Asp Lys Lys Pro Thr Ser Thr Lys Pro Ser Ser Ser Ala  
 1125 1130 1135  
 Pro Arg Val Ser Arg Leu Ala Thr Thr Val Ser Ala Pro Asp Leu Lys  
 1140 1145 1150  
 Ser Val Arg Ser Lys Val Gly Ser Thr Glu Asn Ile Lys His Gln Pro  
 1155 1160 1165  
 Gly Gly Gly Arg Ala Lys Val Glu Lys Lys Thr Glu Ala Ala Thr Thr  
 1170 1175 1180  
 Ala Gly Lys Pro Glu Pro Asn Ala Val Thr Lys Ala Ala Gly Ser Ile  
 1185 1190 1195 1200  
 Ala Ser Ala Gln Lys Pro Pro Ala Gly Lys Val Gln Ile Val Ser Lys  
 1205 1210 1215  
 Lys Val Ser Tyr Ser His Ile Gln Ser Lys Cys Val Ser Lys Asp Asn  
 1220 1225 1230  
 Ile Lys His Val Pro Gly Cys Gly Asn Val Gln Ile Gln Asn Lys Lys  
 1235 1240 1245  
 Val Asp Ile Ser Lys Val Ser Ser Lys Cys Gly Ser Lys Ala Asn Ile  
 1250 1255 1260  
 Lys His Lys Pro Gly Gly Gly Asp Val Lys Ile Glu Ser Gln Lys Leu  
 1265 1270 1275 1280  
 Asn Phe Lys Glu Lys Ala Gln Ala Lys Val Gly Ser Leu Asp Asn Val  
 1285 1290 1295  
 Gly His Phe Pro Ala Gly Gly Ala Val Lys Thr Glu Gly Gly Gly Ser  
 1300 1305 1310

Glu Ala Leu Pro Cys Pro Gly Pro Pro Ala Gly Glu Glu Pro Val Ile  
 1315 1320 1325  
 Pro Glu Ala Ala Pro Asp Arg Gly Ala Pro Thr Ser Ala Ser Gly Leu  
 1330 1335 1340  
 Ser Gly His Thr Thr Leu Ser Gly Gly Gly Asp Gln Arg Glu Pro Gln  
 1345 1350 1355 1360  
 Thr Leu Asp Ser Gln Ile Gln Glu Thr Ser Ile Met Val Ser Lys Gly  
 1365 1370 1375  
 Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly  
 1380 1385 1390  
 Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp  
 1395 1400 1405  
 Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys  
 1410 1415 1420  
 Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr His Gly Val  
 1425 1430 1435 1440  
 Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe  
 1445 1450 1455  
 Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe  
 1460 1465 1470  
 Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly  
 1475 1480 1485  
 Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu  
 1490 1495 1500  
 Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Phe Asn Ser His  
 1505 1510 1515 1520  
 Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn  
 1525 1530 1535  
 Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp  
 1540 1545 1550  
 His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro  
 1555 1560 1565  
 Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn  
 1570 1575 1580  
 Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly  
 1585 1590 1595 1600  
 Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys  
 1605 1610

<210> 23  
 <211> 978  
 <212> DNA

## &lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(978)

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:

GFP-nucleolus-Caspase 8-annexin II construct

&lt;400&gt; 23

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt	48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	

195	200	205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt			672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe			
210	215	220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc			720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser			
225	230	235	240
gga aga aaa cgt ata cgt act tac ctc aag tcc tgc agg cgg atg aaa			768
Gly Arg Lys Arg Ile Arg Thr Tyr Leu Lys Ser Cys Arg Arg Met Lys			
	245	250	255
aga agt ggt ttt gag atg tct cga cct att cct tcc cac ctt act cga			816
Arg Ser Gly Phe Glu Met Ser Arg Pro Ile Pro Ser His Leu Thr Arg			
	260	265	270
tcg gca ggt gtt gaa aca gac gca ggt gtt gaa aca gac gca ggt gtt			864
Ser Ala Gly Val Glu Thr Asp Ala Gly Val Glu Thr Asp Ala Gly Val			
	275	280	285
gaa aca gac gca ggt gtt gaa aca gac gca ggt agt act atg tct act			912
Glu Thr Asp Ala Gly Val Glu Thr Asp Ala Gly Ser Thr Met Ser Thr			
	290	295	300
gtc cac gaa atc ctg tgc aag ctc agc ttg gag ggt gtt cat tct aca			960
Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val His Ser Thr			
305	310	315	320
ccc cca agt gcc gga tcc			978
Pro Pro Ser Ala Gly Ser			
	325		

&lt;210&gt; 24

&lt;211&gt; 326

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:

GFP-nucleolus-Caspase 8-annexin II construct

&lt;400&gt; 24

Met	Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu
1				5					10					15	

Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly
		20						25					30		

Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile
		35					40					45			

Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr
	50					55					60				

Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys
65					70					75					80

Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

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<210> 25
<211> 948
<212> DNA
<213> Artificial Sequence
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<220>  
<221> CDS  
<222> (1) .. (948)
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<220>  
<223> Description of Artificial Sequence:  
GFP-nucleolus-Caspase 3-annexin II construct

<400> 25  
 atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48  
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240  
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288  
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa 336  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt 384  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac 432  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat 480  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160

gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528  
 Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175

gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190

cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt 624  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205

tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt 672  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220

gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc 720  
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser  
 225 230 235 240



gga aga aaa cgt ata cgt act tac ctc aag tcc tgc agg cgg atg aaa 768  
 Gly Arg Lys Arg Ile Arg Thr Tyr Leu Lys Ser Cys Arg Arg Met Lys  
 245 250 255

aga agt ggt ttt gag atg tct cga cct att cct tcc cac ctt act cga 816  
 Arg Ser Gly Phe Glu Met Ser Arg Pro Ile Pro Ser His Leu Thr Arg  
 260 265 270

tcg tat gaa aaa gga ata cca gtt gaa aca gac agc gaa gag caa gct 864  
 Ser Tyr Glu Lys Gly Ile Pro Val Glu Thr Asp Ser Glu Glu Gln Ala  
 275 280 285

tat agt act atg tct act gtc cac gaa atc ctg tgc aag ctc agc ttg 912  
 Tyr Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu  
 290 295 300

gag ggt gtt cat tct aca ccc cca agt gcc gga tcc 948  
 Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser  
 305 310 315

<210> 26

<211> 316

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

GFP-nucleolus-Caspase 3-annexin II construct

<400> 26

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser  
 225 230 235 240  
 Gly Arg Lys Arg Ile Arg Thr Tyr Leu Lys Ser Cys Arg Arg Met Lys  
 245 250 255  
 Arg Ser Gly Phe Glu Met Ser Arg Pro Ile Pro Ser His Leu Thr Arg  
 260 265 270  
 Ser Tyr Glu Lys Gly Ile Pro Val Glu Thr Asp Ser Glu Glu Gln Ala  
 275 280 285  
 Tyr Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu  
 290 295 300  
 Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser  
 305 310 315

<210> 27  
 <211> 2088  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1)..(1041)

<220>  
 <223> Description of Artificial Sequence:  
 NLS-Fred25-synaptobrevin construct

<400> 27  
 atg aga aga aaa cga caa aag gct agc aaa gga gaa gaa ctc ttc act 48  
 Met Arg Arg Lys Arg Gln Lys Ala Ser Lys Gly Glu Glu Leu Phe Thr  
 1 5 10 15  
 gga gtt gtc cca att ctt gtt gaa tta gat ggt gat gtt aac ggc cac 96  
 Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His  
 20 25 30  
 aag ttc tct gtc agt gga gag ggt gaa ggt gat gca aca tac gga aaa 144  
 Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys  
 35 40 45  
 ctt acc cug --- ttc atc tgc act act ggc aaa ctg cct gtt cca ---  
 Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp  
 50 55 60

cca aca cta gtc act act ctg tgc tat ggt gtt caa tgc ttt tca aga	240
Pro Thr Leu Val Thr Thr Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg	
65 70 75 80	
tac ccg gat cat atg aaa cgg cat gac ttt ttc aag agt gcc atg ccc	288
Tyr Pro Asp His Met Lys Arg His Asp Phe Phe Lys Ser Ala Met Pro	
85 90 95	
gaa ggt tat gta cag gaa agg acc atc ttc ttc aaa gat gac ggc aac	336
Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn	
100 105 110	
tac aag aca cgt gct gaa gtc aag ttt gaa ggt gat acc ctt gtt aat	384
Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn	
115 120 125	
aga atc gag tta aaa ggt att gac ttc aag gaa gat ggc aac att ctg	432
Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu	
130 135 140	
gga cac aaa ttg gaa tac aac tat aac tca cac aat gta tac atc atg	480
Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met	
145 150 155 160	
gca gac aaa caa aag aat gga atc aaa gtg aac ttc aag acc cgc cac	528
Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Thr Arg His	
165 170 175	
aac att gaa gat gga agc gtt caa cta gca gac cat tat caa caa aat	576
Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn	
180 185 190	
act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac ctg	624
Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu	
195 200 205	
tcc aca caa tct gcc ctt tcg aaa gat ccc aac gaa aag aga gac cac	672
Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His	
210 215 220	
atg gtc ctt ctt gag ttt gta aca gct gct ggg att aca cat ggc atg	720
Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met	
225 230 235 240	
gat gaa ctg tac aac acc ggt atg tct aca ggt cca act gct gcc act	768
Asp Glu Leu Tyr Asn Thr Gly Met Ser Thr Gly Pro Thr Ala Ala Thr	
245 250 255	
ggc agt aat cga aga ctt cag cag aca caa aat caa gta gat gag gtg	816
Gly Ser Asn Arg Arg Leu Gln Gln Thr Gln Asn Gln Val Asp Glu Val	
260 265 270	
gtg gac ata atg cga gtt aac gtg gac aag gtt ctg gaa aga gac cag	864
Val Asp Ile Met Arg Val Asn Val Asp Lys Val Leu Glu Arg Asp Gln	
275 280 285	
aag ctc tct gag tta gac gac cgt gca gac gca ctg cag gca ggc gct	912
Lys Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala	
290 295 300	
tct caa ttt gaa acg agc gca gcc aag ttg aag agg aaa tat tgg tgg	960

Ser Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp  
 305 310 315 320  
 aag aat tgc aag atg tgg gca atc ggg att act gtt ctg gtt atc ttc 1008  
 Lys Asn Cys Lys Met Trp Ala Ile Gly Ile Thr Val Leu Val Ile Phe  
 325 330 335  
 atc atc atc atc atc gtg tgg gtt gtc tct tca tgaatgagaa gaaaacgaca 1061  
 Ile Ile Ile Ile Ile Val Trp Val Val Ser Ser  
 340 345  
 aaaggctagc aaaggagaag aactcttcac tggagttgtc ccaattcttg ttgaattaga 1121  
 tgggtgatgtt aacggccaca agttctctgt cagtggagag ggtgaagggtg atgcaacata 1181  
 cggaaaactt accctgaagt tcactctgcac tactggcaaa ctgcctgttc catggccaac 1241  
 actagtcact actctgtgct atggtgttca atgcttttca agatacccg atcatatgaa 1301  
 acggcatgac tttttcaaga gtgccatgcc cgaagggttat gtacaggaaa ggaccatctt 1361  
 cttcaaagat gacggcaact acaagacacg tgctgaagtc aagtttgaag gtgataccct 1421  
 tgttaataga atcgagttaa aagggtattga cttcaaggaa gatggcaaca ttctgggaca 1481  
 caaattggaa tacaactata actcacacaa tgtatacatc atggcagaca aaaaaagaa 1541  
 tggaatcaaa gtgaacttca agaccgcga caacattgaa gatggaagcg ttcaactagc 1601  
 agaccattat caacaaaata ctccaattgg cgatggccct gtccttttac cagacaacca 1661  
 ttacctgtcc acacaatctg ccctttcgaa agatcccaac gaaaagagag accacatggt 1721  
 cttctctgag tttgtaacag ctgctgggat tacacatggc atggatgaac tgtacaacac 1781  
 cggtatgtct acaggtccaa ctgctgccac tggcagtaat cgaagacttc agcagacaca 1841  
 aaatcaagta gatgaggtgg tggacataat gcgagttaac gtggacaagg ttctggaaag 1901  
 agaccagaag ctctctgagt tagacgaccg tgcagacgca ctgcaggcag gcgcttctca 1961  
 atttgaaacg agcgcagcca agttgaagag gaaatattgg tggaagaatt gcaagatgtg 2021  
 ggcaatcggg attactgttc tggttatctt catcatcatc atcatcgtgt gggttgtctc 2081  
 ttcatga 2088

&lt;210&gt; 28

&lt;211&gt; 347

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

 <223> Description of Artificial Sequence:  
 NLS-Fred25-synaptobrevin construct

&lt;400&gt; 28

Met Arg Arg Lys Arg Gln Lys Ala Ser Lys Gly Glu Glu Leu Phe Thr  
 1 5 10 15

Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His  
 20 25 30  
 Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys  
 35 40 45  
 Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp  
 50 55 60  
 Pro Thr Leu Val Thr Thr Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg  
 65 70 75 80  
 Tyr Pro Asp His Met Lys Arg His Asp Phe Phe Lys Ser Ala Met Pro  
 85 90 95  
 Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn  
 100 105 110  
 Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn  
 115 120 125  
 Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu  
 130 135 140  
 Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met  
 145 150 155 160  
 Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Thr Arg His  
 165 170 175  
 Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn  
 180 185 190  
 Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu  
 195 200 205  
 Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His  
 210 215 220  
 Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met  
 225 230 235 240  
 Asp Glu Leu Tyr Asn Thr Gly Met Ser Thr Gly Pro Thr Ala Ala Thr  
 245 250 255  
 Gly Ser Asn Arg Arg Leu Gln Gln Thr Gln Asn Gln Val Asp Glu Val  
 260 265 270  
 Val Asp Ile Met Arg Val Asn Val Asp Lys Val Leu Glu Arg Asp Gln  
 275 280 285  
 Lys Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala  
 290 295 300  
 Ser Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp  
 305 310 315 320  
 Lys Asn Cys Lys Met Trp Ala Ile Gly Ile Thr Val Leu Val Ile Phe  
 325 330 335  
 Ile Ile Ile Ile Ile Val Trp Val Val Ser Ser

340

345

<210> 29  
 <211> 2106  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1)..(1050)

<220>  
 <223> Description of Artificial Sequence:  
 NLS-Fred25-cellubrevin construct

<400> 29  
 atg aga aga aaa cga caa aag gct agc aaa gga gaa gaa ctc ttc act 48  
 Met Arg Arg Lys Arg Gln Lys Ala Ser Lys Gly Glu Glu Leu Phe Thr  
 1 5 10 15  
 gga gtt gtc cca att ctt gtt gaa tta gat ggt gat gtt aac ggc cac 96  
 Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His  
 20 25 30  
 aag ttc tct gtc agt gga gag ggt gaa ggt gat gca aca tac gga aaa 144  
 Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys  
 35 40 45  
 ctt acc ctg aag ttc atc tgc act act ggc aaa ctg cct gtt cca tgg 192  
 Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp  
 50 55 60  
 cca aca cta gtc act act ctg tgc tat ggt gtt caa tgc ttt tca aga 240  
 Pro Thr Leu Val Thr Thr Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg  
 65 70 75 80  
 tac ccg gat cat atg aaa cgg cat gac ttt ttc aag agt gcc atg ccc 288  
 Tyr Pro Asp His Met Lys Arg His Asp Phe Phe Lys Ser Ala Met Pro  
 85 90 95  
 gaa ggt tat gta cag gaa agg acc atc ttc ttc aaa gat gac ggc aac 336  
 Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn  
 100 105 110  
 tac aag aca cgt gct gaa gtc aag ttt gaa ggt gat acc ctt gtt aat 384  
 Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn  
 115 120 125  
 aga atc gag tta aaa ggt att gac ttc aag gaa gat ggc aac att ctg 432  
 Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu  
 130 135 140  
 gga cac aaa ttg gaa tac aac tat aac tca cac aat gta tac atc atg 480  
 Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met  
 145 150 155 160  
 gca gac aaa caa aag aat gga atc aaa gtg aac ttc aag acc cgc cac 528  
 Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Thr Arg His  
 165 170 175

aac att gaa gat gga agc gtt caa cta gca gac cat tat caa caa aat 576  
 Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn  
 180 185 190

act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac ctg 624  
 Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu  
 195 200 205

tcc aca caa tct gcc ctt tcg aaa gat ccc aac gaa aag aga gac cac 672  
 Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His  
 210 215 220

atg gtc ctt ctt gag ttt gta aca gct gct ggg att aca cat ggc atg 720  
 Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met  
 225 230 235 240

gat gaa ctg tac aac acc ggt atg tct aca ggt gtg cct tcg ggg tca 768  
 Asp Glu Leu Tyr Asn Thr Gly Met Ser Thr Gly Val Pro Ser Gly Ser  
 245 250 255

agt gct gcc act ggc agt aat cga aga ctc cag cag aca caa aat caa 816  
 Ser Ala Ala Thr Gly Ser Asn Arg Arg Leu Gln Gln Thr Gln Asn Gln  
 260 265 270

gta gat gag gtg gtt gac atc atg aga gtc aat gtg gat aag gtg tta 864  
 Val Asp Glu Val Val Asp Ile Met Arg Val Asn Val Asp Lys Val Leu  
 275 280 285

gaa aga gac cag aag ctc tcg gag cta gat gac cgc gca gat gca ctg 912  
 Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu  
 290 295 300

cag gca ggt gcc tcg cag ttt gaa aca agt gct gcc aag ttg aag aga 960  
 Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys Arg  
 305 310 315 320

aag tat tgg tgg aag aac tgc aag atg tgg gcg ata ggg atc agt gtc 1008  
 Lys Tyr Trp Trp Lys Asn Cys Lys Met Trp Ala Ile Gly Ile Ser Val  
 325 330 335

ctg gtg atc att gtc atc atc atc atc atc gtg tgg tgt gtc tct 1050  
 Leu Val Ile Ile Val Ile Ile Ile Ile Val Trp Cys Val Ser  
 340 345 350

taaatgagaa gaaaacgaca aaaggctagc aaaggagaag aactcttcac tggagttgtc 1110

ccaattcttg ttgaattaga tgggtgatgtt aacggccaca agttctctgt cagtggagag 1170

ggtgaagggtg atgcaacata cggaaaactt accctgaagt tcctctgcac tactggcaaa 1230

ctgcctgttc catggccaac actagtcact actctgtgct atggtgttca atgctttttca 1290

agatacccggt atcatatgaa acggcatgac tttttcaaga gtgccatgcc cgaagggttat 1350

gtacaggaaa ggaccatctt cttcaaagat gacggcaact acaagacacg tgctgaagtc 1410

aagtttgaag gtgataccct tgttaataga atcgagttaa aaggatttga cttcaaggaa 1470

gatggcaaca ttctgggaca caaattggaa tacaactata actcacacaa tgtatacatc 1530

atggcagaca aacaaaagaa tggatcaaaa gtgaacttca agcccgcca caacattgaa 1590

gatggaagcg ttcaactagc agaccattat caacaaaata ctccaattgg cgatggccct 1650  
 gtccttttac cagacaacca ttacctgtcc acacaatctg ccctttcgaa agatcccaac 1710  
 gaaaagagag accacatggg ccttcttgag tttgtaacag ctgctgggat tacacatggc 1770  
 atggatgaac tgtacaacac cggtatgtct acaggtgtgc cttcgggggc aagtgtgcc 1830  
 actggcagta atcgaagact ccagcagaca caaatcaag tagatgaggt ggttgacatc 1890  
 atgagagtca atgtggataa ggtgttagaa agagaccaga agctctcgga gctagatgac 1950  
 cgcgagatg cactgcaggc aggtgcctcg cagtttgaaa caagtgtgc caagttgaag 2010  
 agaaagtatt ggtggaagaa ctgcaagatg tgggcgatag ggatcagtg cctgggtgatc 2070  
 attgtcatca tcatcatcgt gtggtgtgtc tcttaa 2106

<210> 30

<211> 350

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
NLS-Fred25-cellubrevin construct

<400> 30

Met	Arg	Arg	Lys	Arg	Gln	Lys	Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	1	5	10	15
Gly	Val	Val	Pro	Ile	Leu	Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	20	25	30	
Lys	Phe	Ser	Val	Ser	Gly	Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	35	40	45	
Leu	Thr	Leu	Lys	Phe	Ile	Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	50	55	60	
Pro	Thr	Leu	Val	Thr	Thr	Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	65	70	75	80
Tyr	Pro	Asp	His	Met	Lys	Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	85	90	95	
Glu	Gly	Tyr	Val	Gln	Glu	Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	100	105	110	
Tyr	Lys	Thr	Arg	Ala	Glu	Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	115	120	125	
Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	130	135	140	
Gly	His	Lys	Leu	Glu	Tyr	Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	145	150	155	160
Ala	Asp	Lys	Gln	Lys	Asn	Gly	Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His				



<400> 31																	
atg	agg	ccc	aga	aga	aag	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	48	
Met	Arg	Pro	Arg	Arg	Lys	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly		
1				5					10					15			
gtg	gtg	ccc	atc	ctg	gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	96	
Val	Val	Pro	Ile	Leu	Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys		
			20					25					30				
ttc	agc	gtg	tcc	ggc	gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	144	
Phe	Ser	Val	Ser	Gly	Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu		
		35					40					45					

acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc	192
Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro	
50 55 60	
acc ctc gtg acc acc ttc ggc tac ggc ctg cag tgc ttc gcc cgc tac	240
Thr Leu Val Thr Thr Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr	
65 70 75 80	
ccc gac cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa	288
Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu	
85 90 95	
ggc tac gtc cag gag cgc acc atc ttc ttc aag gac gac ggc aac tac	336
Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr	
100 105 110	
aag acc cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc	384
Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg	
115 120 125	
atc gag ctg aag ggc atc gac ttc aag gag gac ggc aac atc ctg ggg	432
Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly	
130 135 140	
cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc atg gcc	480
His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala	
145 150 155 160	
gac aag cag aag aac ggc atc aag gtg aac ttc aag atc cgc cac aac	528
Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn	
165 170 175	
atc gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc	576
Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr	
180 185 190	
ccc atc ggc gac ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc	624
Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser	
195 200 205	
tac cag tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac atg	672
Tyr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met	
210 215 220	
gtc ctg ctg gag ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac	720
Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp	
225 230 235 240	
gag ctg tac aag aag gga gac gaa gtg gac gga gcc gac ctc agt ctt	768
Glu Leu Tyr Lys Lys Gly Asp Glu Val Asp Gly Ala Asp Leu Ser Leu	
245 250 255	
gtg gat gcg ttg aca gaa cca cct cca gaa att gag gga gaa ata aag	816
Val Asp Ala Leu Thr Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys	
260 265 270	
cga gac ttc atg gct gcg ctg gag gca gag ccc tat gat gac atc gtg	864
Arg Asp Phe Met Ala Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val	
275 280 285	

gga gaa act gtg gag aaa act gag ttt att cct ctc ctg gat ggt gat	912
Gly Glu Thr Val Glu Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp	
290 295 300	
gag aaa acc ggg aac tca gag tcc aaa aag aaa ccc tgc tta gac act	960
Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr	
305 310 315 320	
agc cag gtt gaa ggt atc cca tct tct aaa cca aca ctc cta gcc aat	1008
Ser Gln Val Glu Gly Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn	
325 330 335	
ggt gat cat gga atg gag ggg aat aac act gca ggg tct cca act gac	1056
Gly Asp His Gly Met Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp	
340 345 350	
ttc ctt gaa gag aga gtg gac tat ccg gat tat cag agc agc cag aac	1104
Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn	
355 360 365	
tgg cca gaa gat gca agc ttt tgt ttc cag cct cag caa gtg tta gat	1152
Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp	
370 375 380	
act gac cag gct gag ccc ttt aac gag cac cgt gat gat ggt ttg gca	1200
Thr Asp Gln Ala Glu Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala	
385 390 395 400	
gat ctg ctc ttt gtc tcc agt gga ccc acg aac gct tct gca ttt aca	1248
Asp Leu Leu Phe Val Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr	
405 410 415	
gag cga gac aat cct tca gaa gac agt tac ggt atg ctt ccc tgt gac	1296
Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp	
420 425 430	
tca ttt gct tcc acg gct gtt gta tct cag gag tgg tct gtg gga gcc	1344
Ser Phe Ala Ser Thr Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala	
435 440 445	
cca aac tct cca tgt tca gag tcc tgt gtc tcc cca gag gtt act ata	1392
Pro Asn Ser Pro Cys Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile	
450 455 460	
gaa acc cta cag cca gca aca gag ctc tcc aag gca gca gaa gtg gaa	1440
Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu	
465 470 475 480	
tca gtg aaa gag cag ctg cca gct aaa gca ttg gaa acg atg gca gag	1488
Ser Val Lys Glu Gln Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu	
485 490 495	
cag acc act gat gtg gtg cac tct cca tcc aca gac aca aca cca ggc	1536
Gln Thr Thr Asp Val Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly	
500 505 510	
cca gac aca gag gca gca ctg gct aaa gac ata gaa gag atc acc aag	1584
Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys	
515 520 525	
cca gat gtg ata ttg gca aat gtc acg cag cca tct act gaa tcg gat	1632

Pro	Asp	Val	Ile	Leu	Ala	Asn	Val	Thr	Gln	Pro	Ser	Thr	Glu	Ser	Asp	
530						535					540					
atg	ttc	ctg	gcc	cag	gac	atg	gaa	cta	ctc	aca	gga	aca	gag	gca	gcc	1680
Met	Phe	Leu	Ala	Gln	Asp	Met	Glu	Leu	Leu	Thr	Gly	Thr	Glu	Ala	Ala	
545					550					555					560	
cac	gct	aac	aat	atc	ata	ttg	cct	aca	gaa	cca	gac	gaa	tct	tca	acc	1728
His	Ala	Asn	Asn	Ile	Ile	Leu	Pro	Thr	Glu	Pro	Asp	Glu	Ser	Ser	Thr	
				565					570						575	
aag	gat	gta	gca	cca	cct	atg	gaa	gaa	gaa	att	gtc	cca	ggc	aat	gat	1776
Lys	Asp	Val	Ala	Pro	Pro	Met	Glu	Glu	Glu	Ile	Val	Pro	Gly	Asn	Asp	
			580					585					590			
acg	aca	tcc	ccc	aaa	gaa	aca	gag	aca	aca	ctt	cca	ata	aaa	atg	gac	1824
Thr	Thr	Ser	Pro	Lys	Glu	Thr	Glu	Thr	Thr	Leu	Pro	Ile	Lys	Met	Asp	
		595					600					605				
ttg	gca	cca	cct	gag	gat	gtg	tta	ctt	acc	aaa	gaa	aca	gaa	cta	gcc	1872
Leu	Ala	Pro	Pro	Glu	Asp	Val	Leu	Leu	Thr	Lys	Glu	Thr	Glu	Leu	Ala	
	610					615					620					
cca	gcc	aag	ggc	atg	gtt	tca	ctc	tca	gaa	ata	gaa	gag	gct	ctg	gca	1920
Pro	Ala	Lys	Gly	Met	Val	Ser	Leu	Ser	Glu	Ile	Glu	Glu	Ala	Leu	Ala	
625					630					635					640	
aag	aat	gat	gtt	cgc	tct	gca	gaa	ata	cct	gtg	gct	cag	gag	aca	gtg	1968
Lys	Asn	Asp	Val	Arg	Ser	Ala	Glu	Ile	Pro	Val	Ala	Gln	Glu	Thr	Val	
				645					650						655	
gtc	tca	gaa	aca	gag	gtg	gtc	ctg	gca	aca	gaa	gtg	gta	ctg	ccc	tca	2016
Val	Ser	Glu	Thr	Glu	Val	Val	Leu	Ala	Thr	Glu	Val	Val	Leu	Pro	Ser	
			660					665					670			
gat	ccc	ata	aca	aca	ttg	aca	aag	gat	gtg	aca	ctc	ccc	tta	gaa	gca	2064
Asp	Pro	Ile	Thr	Thr	Leu	Thr	Lys	Asp	Val	Thr	Leu	Pro	Leu	Glu	Ala	
		675					680					685				
gag	aga	ccg	ttg	gtg	acg	gac	atg	act	cca	tct	ctg	gaa	aca	gaa	atg	2112
Glu	Arg	Pro	Leu	Val	Thr	Asp	Met	Thr	Pro	Ser	Leu	Glu	Thr	Glu	Met	
	690					695					700					
acc	cta	ggc	aaa	gag	aca	gct	cca	ccc	aca	gaa	aca	aat	ttg	ggc	atg	2160
Thr	Leu	Gly	Lys	Glu	Thr	Ala	Pro	Pro	Thr	Glu	Thr	Asn	Leu	Gly	Met	
705					710					715					720	
gcc	aaa	gac	atg	tct	cca	ctc	cca	gaa	tca	gaa	gtg	act	ctg	ggc	aag	2208
Ala	Lys	Asp	Met	Ser	Pro	Leu	Pro	Glu	Ser	Glu	Val	Thr	Leu	Gly	Lys	
				725					730					735		
gac	gtg	gtt	ata	ctt	cca	gaa	aca	aag	gtg	gct	gag	ttt	aac	aat	gtg	2256
Asp	Val	Val	Ile	Leu	Pro	Glu	Thr	Lys	Val	Ala	Glu	Phe	Asn	Asn	Val	
			740					745					750			
act	cca	ctt	tca	gaa	gaa	gag	gta	acc	tca	gtc	aag	gac	atg	tct	ccg	2304
Thr	Pro	Leu	Ser	Glu	Glu	Glu	Val	Thr	Ser	Val	Lys	Asp	Met	Ser	Pro	
		755					760					765				
tct	gca	gaa	aca	gag	gct	ccc	ctg	gct	aag	aat	gct	gat	ctg	cac	tca	2352
Ser	Ala	Glu	Thr	Glu	Ala	Pro	Leu	Ala	Lys	Asn	Ala	Asp	Leu	His	Ser	

770	775	780	
gga aca gag ctg att gtg gac aac agc atg gct cca gcc tcc gat ctt Gly Thr Glu Leu Ile Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu 785 790 795 800			2400
gca ctg ccc ttg gaa aca aaa gta gca aca gtt cca att aaa gac aaa Ala Leu Pro Leu Glu Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys 805 810 815			2448
gga atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc Gly Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile 820 825 830			2496
ctg gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser 835 840 845			2544
ggc gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe 850 855 860			2592
atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr 865 870 875 880			2640
acc ctg acc cac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg Thr Leu Thr His Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met 885 890 895			2688
aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln 900 905 910			2736
gag cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala 915 920 925			2784
gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys 930 935 940			2832
ggc atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu 945 950 955 960			2880
tac aac ttc aac agc cac aac gtc tat atc atg gcc gac aag cag aag Tyr Asn Phe Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys 965 970 975			2928
aac ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly 980 985 990			2976
agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp 995 1000 1005			3024
ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala 1010 1015 1020			3072

ctg agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag 3120  
 Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu  
 1025 1030 1035 1040

ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag 3168  
 Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys  
 1045 1050 1055

tag 3171

<210> 32

<211> 1056

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

NLS-EYFP-MAPKDM-EBFP construct

<400> 32

Met Arg Pro Arg Arg Lys Val Ser Lys Gly Glu Glu Leu Phe Thr Gly  
 1 5 10 15

Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys  
 20 25 30

Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu  
 35 40 45

Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro  
 50 55 60

Thr Leu Val Thr Thr Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr  
 65 70 75 80

Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu  
 85 90 95

Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr  
 100 105 110

Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg  
 115 120 125

Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly  
 130 135 140

His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala  
 145 150 155 160

Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn  
 165 170 175

Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr  
 180 185 190

Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser  
 195 200 205

Tyr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met  
 210 215 220  
 Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp  
 225 230 235 240  
 Glu Leu Tyr Lys Lys Gly Asp Glu Val Asp Gly Ala Asp Leu Ser Leu  
 245 250 255  
 Val Asp Ala Leu Thr Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys  
 260 265 270  
 Arg Asp Phe Met Ala Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val  
 275 280 285  
 Gly Glu Thr Val Glu Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp  
 290 295 300  
 Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr  
 305 310 315 320  
 Ser Gln Val Glu Gly Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn  
 325 330 335  
 Gly Asp His Gly Met Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp  
 340 345 350  
 Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn  
 355 360 365  
 Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp  
 370 375 380  
 Thr Asp Gln Ala Glu Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala  
 385 390 395 400  
 Asp Leu Leu Phe Val Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr  
 405 410 415  
 Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp  
 420 425 430  
 Ser Phe Ala Ser Thr Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala  
 435 440 445  
 Pro Asn Ser Pro Cys Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile  
 450 455 460  
 Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu  
 465 470 475 480  
 Ser Val Lys Glu Gln Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu  
 485 490 495  
 Gln Thr Thr Asp Val Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly  
 500 505 510  
 Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys  
 515 520 525  
 Pro Asp Val Ile Leu Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp

530					535					540					
Met	Phe	Leu	Ala	Gln	Asp	Met	Glu	Leu	Leu	Thr	Gly	Thr	Glu	Ala	Ala
545					550					555					560
His	Ala	Asn	Asn	Ile	Ile	Leu	Pro	Thr	Glu	Pro	Asp	Glu	Ser	Ser	Thr
				565					570					575	
Lys	Asp	Val	Ala	Pro	Pro	Met	Glu	Glu	Glu	Ile	Val	Pro	Gly	Asn	Asp
			580					585					590		
Thr	Thr	Ser	Pro	Lys	Glu	Thr	Glu	Thr	Thr	Leu	Pro	Ile	Lys	Met	Asp
		595					600					605			
Leu	Ala	Pro	Pro	Glu	Asp	Val	Leu	Leu	Thr	Lys	Glu	Thr	Glu	Leu	Ala
	610					615					620				
Pro	Ala	Lys	Gly	Met	Val	Ser	Leu	Ser	Glu	Ile	Glu	Glu	Ala	Leu	Ala
625					630					635					640
Lys	Asn	Asp	Val	Arg	Ser	Ala	Glu	Ile	Pro	Val	Ala	Gln	Glu	Thr	Val
				645					650					655	
Val	Ser	Glu	Thr	Glu	Val	Val	Leu	Ala	Thr	Glu	Val	Val	Leu	Pro	Ser
			660					665					670		
Asp	Pro	Ile	Thr	Thr	Leu	Thr	Lys	Asp	Val	Thr	Leu	Pro	Leu	Glu	Ala
		675					680					685			
Glu	Arg	Pro	Leu	Val	Thr	Asp	Met	Thr	Pro	Ser	Leu	Glu	Thr	Glu	Met
	690					695					700				
Thr	Leu	Gly	Lys	Glu	Thr	Ala	Pro	Pro	Thr	Glu	Thr	Asn	Leu	Gly	Met
705					710					715					720
Ala	Lys	Asp	Met	Ser	Pro	Leu	Pro	Glu	Ser	Glu	Val	Thr	Leu	Gly	Lys
				725					730					735	
Asp	Val	Val	Ile	Leu	Pro	Glu	Thr	Lys	Val	Ala	Glu	Phe	Asn	Asn	Val
			740					745					750		
Thr	Pro	Leu	Ser	Glu	Glu	Glu	Val	Thr	Ser	Val	Lys	Asp	Met	Ser	Pro
		755					760					765			
Ser	Ala	Glu	Thr	Glu	Ala	Pro	Leu	Ala	Lys	Asn	Ala	Asp	Leu	His	Ser
	770					775					780				
Gly	Thr	Glu	Leu	Ile	Val	Asp	Asn	Ser	Met	Ala	Pro	Ala	Ser	Asp	Leu
785					790					795					800
Ala	Leu	Pro	Leu	Glu	Thr	Lys	Val	Ala	Thr	Val	Pro	Ile	Lys	Asp	Lys
				805					810					815	
Gly	Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile
			820					825					830		
Leu	Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser
		835					840					845			
Gly	Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe
	850					855					860				



Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr  
 865 870 875 880  
 Thr Leu Thr His Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met  
 885 890 895  
 Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln  
 900 905 910  
 Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala  
 915 920 925  
 Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys  
 930 935 940  
 Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu  
 945 950 955 960  
 Tyr Asn Phe Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys  
 965 970 975  
 Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly  
 980 985 990  
 Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp  
 995 1000 1005  
 Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala  
 1010 1015 1020  
 Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu  
 1025 1030 1035 1040  
 Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys  
 1045 1050 1055

&lt;210&gt; 33

&lt;211&gt; 1623

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(1623)

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:

YFP-NLS-CP3-multiple DEVD-CFP-Annexin II construct

&lt;400&gt; 33

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg 48  
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15  
 gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc 96  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc 144

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ttc ggc tac ggc ctg cag tgc ttc gcc cgc tac ccc gac cac atg aag	240
Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag	288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc tac cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	
225 230 235 240	
gga aga agg aaa cga caa aag cga tgc gca ggt gac gaa gtt gat gca	768
Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Asp Glu Val Asp Ala	
245 250 255	
ggt gac gaa gtt gat gca ggt gac gaa gtt gat gca ggt gac gaa gtt	816
Gly Asp Glu Val Asp Ala Gly Asp Glu Val Asp Ala Gly Asp Glu Val	
260 265 270	
gac gca ggt agt act atg gtg agc aag ggc gag gag ctg ttc acc ggg	864
Asp Ala Gly Ser Thr Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly	

275	280	285	
gtg gtc ccc atc ctg gtc gag ctg gac ggc gac gta aac ggc cac aag			912
Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys			
290	295	300	
ttc agc gtg tcc ggc gag ggc gag ggc gat gcc acc tac ggc aag ctg			960
Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu			
305	310	315	320
acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc			1008
Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro			
325	330	335	
acc ctc gtg acc acc ctg acc tgg ggc gtg cag tgc ttc agc cgc tac			1056
Thr Leu Val Thr Thr Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr			
340	345	350	
ccc gac cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa			1104
Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu			
355	360	365	
ggc tac gtc cag gag cgc acc atc ttc ttc aag gac gac ggc aac tac			1152
Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr			
370	375	380	
aag acc cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc			1200
Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg			
385	390	395	400
atc gag ctg aag ggc atc gac ttc aag gag gac ggc aac atc ctg ggg			1248
Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly			
405	410	415	
cac aag ctg gag tac aac tac atc agc cac aac gtc tat atc acc gcc			1296
His Lys Leu Tyr Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala			
420	425	430	
gac aag cag aag aac ggc atc aag gcc aac ttc aag atc cgc cac aac			1344
Asp Lys Gln Lys Asn Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn			
435	440	445	
atc gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc			1392
Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr			
450	455	460	
ccc atc ggc gac ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc			1440
Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser			
465	470	475	480
acc cag tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac atg			1488
Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met			
485	490	495	
gtc ctg ctg gag ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac			1536
Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp			
500	505	510	
gag ctg tac aag atg tct act gtc cac gaa atc ctg tgc aag ctc agc			1584
Glu Leu Tyr Lys Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser			
515	520	525	

ttg gag ggt gtt cat tct aca ccc cca agt gcc gga tcc  
 Leu Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser  
           530                                  535                                  540

<210> 34  
 <211> 541  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
       YFP-NLS-CP3-multiple DEVD-CFP-Annexin II construct

<400> 34  
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
   1                                  5                                  10                                  15  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
                                   20                                  25                                  30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
                                   35                                  40                                  45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
                                   50                                  55                                  60  
 Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys  
   65                                  70                                  75                                  80  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
                                   85                                  90                                  95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
                                   100                                  105                                  110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
                                   115                                  120                                  125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
   130                                  135                                  140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
   145                                  150                                  155                                  160  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
                                   165                                  170                                  175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
                                   180                                  185                                  190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu  
   195                                  200                                  205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
   210                                  215                                  220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
   225                                  230                                  235                                  240

Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Asp Glu Val Asp Ala  
 245 250 255  
 Gly Asp Glu Val Asp Ala Gly Asp Glu Val Asp Ala Gly Asp Glu Val  
 260 265 270  
 Asp Ala Gly Ser Thr Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly  
 275 280 285  
 Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys  
 290 295 300  
 Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu  
 305 310 315 320  
 Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro  
 325 330 335  
 Thr Leu Val Thr Thr Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr  
 340 345 350  
 Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu  
 355 360 365  
 Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr  
 370 375 380  
 Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg  
 385 390 395 400  
 Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly  
 405 410 415  
 His Lys Leu Glu Tyr Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala  
 420 425 430  
 Asp Lys Gln Lys Asn Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn  
 435 440 445  
 Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr  
 450 455 460  
 Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser  
 465 470 475 480  
 Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met  
 485 490 495  
 Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp  
 500 505 510  
 Glu Leu Tyr Lys Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser  
 515 520 525  
 Leu Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser  
 530 535 540

&lt;210&gt; 35

&lt;211&gt; 24

&lt;212&gt; DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: FLAG epitope

<400> 35

gactacaaag acgacgacga caaa

24

<210> 36

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: FLAG epitope

<400> 36

Asp Tyr Lys Asp Asp Asp Asp Lys

1

5

<210> 37

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HA epitope

<400> 37

taccatacgc acgtaccaga ctacgca

27

<210> 38

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HA epitope

<400> 38

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala

1

5

<210> 39

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: KT3 epitope

<400> 39

ccaccagaac cagaaaca

18

<210> 40

<211> 6



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tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc 192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50 55 60

ttc ggc tac ggc ctg cag tgc ttc gcc cgc tac ccc gac cac atg aag 240
Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
65 70 75 80

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag 288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag 336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110

gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc 384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
115 120 125

atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac 432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
130 135 140

aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac 480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145 150 155 160

ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc 528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
165 170 175

gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc 576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180 185 190

ccc gtg ctg ctg ccc gac aac cac tac ctg agc tac cag tcc gcc ctg 624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu
195 200 205

agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc 672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
210 215 220

gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag 717
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
225 230 235

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&lt;210&gt; 44

&lt;211&gt; 239

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: EYFP

&lt;400&gt; 44

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15

```



Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
                   20                                  25                                  30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
                   35                                  40                                  45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
                   50                                  55                                  60  
 Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys  
                   65                                  70                                  75                                  80  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
                                   85                                  90                                  95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
                                   100                                  105                                  110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
                   115                                  120                                  125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
                   130                                  135                                  140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
                   145                                  150                                  155                                  160  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
                                   165                                  170                                  175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
                   180                                  185                                  190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu  
                   195                                  200                                  205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
                   210                                  215                                  220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys  
                   225                                  230                                  235  
  
 <210> 45  
 <211> 717  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <221> CDS  
 <222> (1)..(717)  
  
 <220>  
 <223> Description of Artificial Sequence: EGFP  
  
 <400> 45  
 atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg 48  
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
   1                                  5                                  10                                  15  
 gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc 96

Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly		
			20					25					30				
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144	
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile		
		35					40				45						
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr		
	50					55					60						
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240	
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys		
65					70				75						80		
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288	
Gln	His	Asp	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu			
			85					90						95			
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu		
			100					105					110				
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly		
		115					120					125					
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr		
	130					135					140						
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn		
145					150					155					160		
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528	
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser		
			165					170						175			
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly		
			180					185					190				
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624	
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu		
		195				200						205					
agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe		
	210					215					220						
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag		717	
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys			
225					230					235							

&lt;210&gt; 46

&lt;211&gt; 239

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: EGFP

&lt;400&gt; 46

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1           5           10           15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
          20           25           30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35           40           45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50           55           60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65           70           75           80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
          85           90           95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
          100          105          110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
          115          120          125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
          130          135          140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
          145          150          155          160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
          165          170          175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
          180          185          190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
          195          200          205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
          210          215          220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
          225          230          235

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&lt;210&gt; 47

&lt;211&gt; 717

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(717)

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: EBFP

78

<210> 48  
 <211> 239  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: EBFP

<400> 48  
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
           1                  5                  10                  15  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
                   20                  25                  30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
                   35                  40                  45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
           50                  55                  60  
 Leu Thr His Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
           65                  70                  75                  80  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
                   85                  90                  95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
                   100                  105                  110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
                   115                  120                  125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
           130                  135                  140  
 Asn Phe Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
           145                  150                  155                  160  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
                   165                  170                  175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
                   180                  185                  190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
                   195                  200                  205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
           210                  215                  220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys  
           225                  230                  235

<210> 49  
 <211> 717  
 <212> DNA  
 <213> Artificial Sequence

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(717)

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: ECFP

&lt;400&gt; 49

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5				10					15			
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
			35				40						45			
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ctg	acc	tgg	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Trp	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
	65				70				75						80	
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
				85					90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
			115				120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
			130			135					140					
aac	tac	atc	agc	cac	aac	gtc	tat	atc	acc	gcc	gac	aag	cag	aag	aac	480
Asn	Tyr	Ile	Ser	His	Asn	Val	Tyr	Ile	Thr	Ala	Asp	Lys	Gln	Lys	Asn	
					150					155					160	
ggc	atc	aag	gcc	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Ala	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
				165				170						175		
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180				185						190			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	
			195			200						205				

agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc 672  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220

gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag 717  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys  
 225 230 235

<210> 50

<211> 239

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: ECFP

<400> 50

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala Asp Lys Gln Lys Asn  
 145 150 155 160

Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr

225

230

235

&lt;210&gt; 51

&lt;211&gt; 720

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(717)

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Fred25

&lt;400&gt; 51

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt	48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
gga atc aaa aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtt caa cta gca gac cat caa caa aat act cca att ggc gat ggc	576



<400> 52																
Met	Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5					10					15		
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
		35					40					45				
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70					75					80	
Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
				85					90					95		
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130					135					140					
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
Gly	Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
				165					170					175		
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180					185					190			

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220

Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn  
 225 230 235

<210> 53

<211> 14

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-1,4,5  
 substrate recognition sequence

<400> 53

tggaacatg acaa

14

<210> 54

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-1,4,5  
 substrate recognition sequence

<400> 54

Trp Glu His Asp

1

<210> 55

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-1  
 substrate recognition sequence

<400> 55

tggtttaaag ac

12

<210> 56

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-1  
 substrate recognition sequence

<400> 56

Trp Phe Lys Asp

1

<210> 57  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-2  
substrate recognition sequence

<400> 57  
gacgaacacg ac

12

<210> 58  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-2  
substrate recognition sequence

<400> 58  
Asp Glu His Asp  
1

<210> 59  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-3,7  
substrate recognition sequence

<400> 59  
gacgaagttg ac

12

<210> 60  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-3,7  
substrate recognition sequence

<400> 60  
Asp Glu Val Asp  
1

<210> 61  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-3  
substrate recognition sequence

<400> 61

atagaaacag ac

12

<210> 62

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-3  
substrate recognition sequence

<400> 62

Ile Glu Thr Asp

1

<210> 63

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-4,5  
substrate recognition sequence

<400> 63

tgggtaagag ac

12

<210> 64

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-4,5  
substrate recognition sequence

<400> 64

Trp Val Arg Asp

1

<210> 65

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-6  
substrate recognition sequence

<400> 65

gtagaaatag ac

12

<210> 66  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-6  
substrate recognition sequence

<400> 66  
Val Glu Ile Asp  
1

<210> 67  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-6  
substrate recognition sequence

<400> 67  
gtagaacacg ac

12

<210> 68  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-6  
substrate recognition sequence

<400> 68  
Val Glu His Asp  
1

<210> 69  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-6  
substrate recognition sequence

<400> 69  
acagaagtag ac

12

<210> 70  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-6  
substrate recognition sequence

<400> 70

Thr Glu Val Asp

1

<210> 71

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-7  
substrate recognition sequence

<400> 71

atacaagcag ac

12

<210> 72

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-7  
substrate recognition sequence

<400> 72

Ile Gln Ala Asp

1

<210> 73

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-8  
substrate recognition sequence

<400> 73

gtagaaacag ac

12

<210> 74

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-8  
substrate recognition sequence

<400> 74

Val Glu Thr Asp

1

<210> 75  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-8  
substrate recognition sequence

<400> 75  
ttagaaacag ac

12

<210> 76  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-8  
substrate recognition sequence

<400> 76  
Leu Glu Thr Asp  
1

<210> 77  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-9  
substrate recognition sequence

<400> 77  
ttagaacacg ac

12

<210> 78  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-9  
substrate recognition sequence

<400> 78  
Leu Glu His Asp  
1

<210> 79  
<211> 12  
<212> DNA  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: proCaspase-9  
substrate recognition sequence

&lt;400&gt; 79

ttagaacacg ac

12

&lt;210&gt; 80

&lt;211&gt; 4

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: proCaspase-9  
substrate recognition sequence

&lt;400&gt; 80

Leu Glu His Asp

1

&lt;210&gt; 81

&lt;211&gt; 12

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: HIV protease  
substrate recognition sequence

&lt;400&gt; 81

agccaaaatt ac

12

&lt;210&gt; 82

&lt;211&gt; 4

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: HIV protease  
substrate recognition sequence

&lt;400&gt; 82

Ser Gln Asn Tyr

1

&lt;210&gt; 83

&lt;211&gt; 12

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: HIV protease  
substrate recognition sequence

&lt;400&gt; 83

ccaatagtag aa

12



<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-2  
substrate recognition sequence

<400> 57  
gacgaacacg ac

12

<210> 58

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-2  
substrate recognition sequence

<400> 58  
Asp Glu His Asp  
1

<210> 59

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-3,7  
substrate recognition sequence

<400> 59  
gacgaagttg ac

12

<210> 60

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-3,7  
substrate recognition sequence

<400> 60  
Asp Glu Val Asp  
1

<210> 61

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-3  
substrate recognition sequence

<400> 61  
atagaaacag ac

12

<210> 62  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-3  
substrate recognition sequence

<400> 62  
Ile Glu Thr Asp  
1

<210> 63  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-4,5  
substrate recognition sequence

<400> 63  
tgggtaagag ac

12

<210> 64  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-4,5  
substrate recognition sequence

<400> 64  
Trp Val Arg Asp  
1

<210> 65  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-6  
substrate recognition sequence

<400> 65  
gtagaaatag ac

12

<210> 66  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-6  
substrate recognition sequence

<400> 66  
Val Glu Ile Asp  
1

<210> 67  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-6  
substrate recognition sequence

<400> 67  
gtagaacacg ac

12

<210> 68  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-6  
substrate recognition sequence

<400> 68  
Val Glu His Asp  
1

<210> 69  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-6  
substrate recognition sequence

<400> 69  
acagaagtag ac

12

<210> 70  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-6  
substrate recognition sequence

<400> 70  
Thr Glu Val Asp  
1

<210> 71  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-7  
substrate recognition sequence

<400> 71  
atacaagcag ac

12

<210> 72  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-7  
substrate recognition sequence

<400> 72  
Ile Gln Ala Asp  
1

<210> 73  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-8  
substrate recognition sequence

<400> 73  
gtagaaacag ac

12

<210> 74  
<211> 4

<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-8  
substrate recognition sequence

<400> 74  
Val Glu Thr Asp  
1

<210> 75  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-8  
substrate recognition sequence

<400> 75  
ttagaaacag ac

12

<210> 76  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: proCaspase-8  
substrate recognition sequence

<400> 76  
Leu Glu Thr Asp  
1

<210> 77  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-9  
substrate recognition sequence

<400> 77  
ttagaacacg ac

12

<210> 78  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-9  
substrate recognition sequence

<400> 78

Leu Glu His Asp

1

<210> 79

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-9  
substrate recognition sequence

<400> 79

ttagaacacg ac

12

<210> 80

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-9  
substrate recognition sequence

<400> 80

Leu Glu His Asp

1

<210> 81

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HIV protease  
substrate recognition sequence

<400> 81

agccaaaatt ac

12

<210> 82

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HIV protease  
substrate recognition sequence

<400> 82  
Ser Gln Asn Tyr  
1

<210> 83  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: HIV protease  
substrate recognition sequence

<400> 83  
ccaatagtac aa

12

<210> 84  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: HIV protease  
substrate recognition sequence

<400> 84  
Pro Ile Val Gln  
1

<210> 85  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Adenovirus  
endopeptidase substrate recognition sequence

<400> 85  
atgttttgag ga

12

<210> 86  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Adenovirus  
endopeptidase substrate recognition sequence

<400> 86  
Met Phe Gly Gly

1

<210> 87  
<211> 12  
<212> DNA  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Adenovirus  
          endopeptidase substrate recognition sequence

<400> 87  
gcaaaaaaaaa ga

12

<210> 88  
<211> 4  
<212> PRT  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Adenovirus  
          endopeptidase substrate recognition sequence

<400> 88  
Ala Lys Lys Arg  
1

<210> 89  
<211> 9  
<212> DNA  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: b-Secretase  
          substrate recognition sequence

<400> 89  
gtgaaaatg

9

<210> 90  
<211> 3  
<212> PRT  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: b-Secretase  
          substrate recognition sequence

<400> 90  
Val Lys Met  
1



<210> 91  
<211> 12  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: b-Secretase  
substrate recognition sequence

<400> 91  
gacgcagaat tc

12

<210> 92  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: b-Secretase  
substrate recognition sequence

<400> 92  
Asp Ala Glu Phe  
1

<210> 93  
<211> 15  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Cathepsin D  
substrate recognition sequence

<400> 93  
aaaccagcat tattc

15

<210> 94  
<211> 5  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Cathepsin D  
substrate recognition sequence

<400> 94  
Lys Pro Ala Leu Phe  
1 5

<210> 95  
<211> 9  
<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Cathepsin D  
substrate recognition sequence

<400> 95  
ttcagatta

9

<210> 96

<211> 3

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Cathepsin D  
substrate recognition sequence

<400> 96  
Phe Arg Leu  
1

<210> 97

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Matrix  
Metalloprotease substrate recognition sequence

<400> 97  
ggaccattag gacca

15

<210> 98

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Matrix  
Metalloprotease substrate recognition sequence

<400> 98  
Gly Pro Leu Gly Pro  
1 5

<210> 99

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Granzyme B  
substrate recognition sequence

<400> 99  
atagaaccag ac

12

<210> 100  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Granzyme B  
substrate recognition sequence

<400> 100  
Ile Glu Pro Asp  
1

<210> 101  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Anthrax  
protease substrate recognition sequence

<400> 101  
atgcccaaga agaagccgac gcccatccag ctgaac

36

<210> 102  
<211> 12  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Anthrax  
protease substrate recognition sequence

<400> 102  
Met Pro Lys Lys Lys Pro Thr Pro Ile Gln Leu Asn  
1 5 10

<210> 103  
<211> 45  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Anthrax  
protease substrate recognition sequence

&lt;400&gt; 103

atgctggccc ggaggaagcc ggtgctgccg gcgctcacca tcaac

45

&lt;210&gt; 104

&lt;211&gt; 15

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Anthrax  
protease substrate recognition sequence

&lt;400&gt; 104

Met Leu Ala Arg Arg Lys Pro Val Leu Pro Ala Leu Thr Ile Asn  
1 5 10 15

&lt;210&gt; 105

&lt;211&gt; 18

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:  
tetanus/botulium substrate recognition sequence

&lt;400&gt; 105

gcctcgcagt ttgaaaca

18

&lt;210&gt; 106

&lt;211&gt; 6

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:  
tetanus/botulium substrate recognition sequence

&lt;400&gt; 106

Ala Ser Gln Phe Glu Thr  
1 5

&lt;210&gt; 107

&lt;211&gt; 18

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:  
tetanus/botulium substrate recognition sequence

&lt;400&gt; 107

gcttctcaat ttgaaacg

18

<210> 108  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
tetanus/botulium substrate recognition sequence

<400> 108  
Ala Ser Gln Phe Glu Thr  
1 5

<210> 109  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin A substrate recognition sequence

<400> 109  
gccaaccaac gtgcaaca

18

<210> 110  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin A substrate recognition sequence

<400> 110  
Ala Asn Gln Arg Ala Thr  
1 5

<210> 111  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin B substrate recognition sequence

<400> 111  
gcttctcaat ttgaaacg

18

<210> 112  
<211> 6

<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin B substrate recognition sequence

<400> 112  
Ala Ser Gln Phe Glu Thr  
1 5

<210> 113  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin C substrate recognition sequence

<400> 113  
acgaaaaaag ctgtgaaa

18

<210> 114  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin C substrate recognition sequence

<400> 114  
Thr Lys Lys Ala Val Lys  
1 5

<210> 115  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin D substrate recognition sequence

<400> 115  
gaccagaagc tctctgag

18

<210> 116  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum  
neurotoxin D substrate recognition sequence

<400> 116

Asp Gln Lys Leu Ser Glu  
1 5

<210> 117

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum  
neurotoxin E substrate recognition sequence

<400> 117

atcgacagga tcatggag

18

<210> 118

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum  
neurotoxin E substrate recognition sequence

<400> 118

Ile Asp Arg Ile Met Glu  
1 5

<210> 119

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum  
neurotoxin F substrate recognition sequence

<400> 119

agagaccaga agctctct

18

<210> 120

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum  
neurotoxin F substrate recognition sequence

<400> 120  
Arg Asp Gln Lys Leu Ser  
1 5

<210> 121  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin G substrate recognition sequence

<400> 121  
acgagcgcag ccaagttg

18

<210> 122  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Botulinum  
neurotoxin G substrate recognition sequence

<400> 122  
Thr Ser Ala Ala Lys Leu  
1 5

<210> 123  
<211> 69  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Cytoplasm/cytoskeleton target sequence

<400> 123  
atgtctactg tccacgaaat cctgtgcaag ctcagcttgg aggggtgttca ttctacaccc 60  
ccaagtgcc

69

<210> 124  
<211> 23  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Cytoplasm/cytoskeleton target sequence



&lt;400&gt; 124

Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val  
 1 5 10 15

His Ser Thr Pro Pro Ser Ala  
 20

&lt;210&gt; 125

&lt;211&gt; 96

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Inner surface  
 of plasma membrane target sequence

&lt;400&gt; 125

atgggatgta cattaagcgc agaagacaaa gcagcagtag aaagaagcaa aatgatagac 60  
 agaaacttaa gagaagacgg agaaaaagct gctaga 96

&lt;210&gt; 126

&lt;211&gt; 32

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Inner surface  
 of plasma membrane target sequence

&lt;400&gt; 126

Met Gly Cys Thr Leu Ser Ala Glu Asp Lys Ala Ala Val Glu Arg Ser  
 1 5 10 15

Lys Met Ile Asp Arg Asn Leu Arg Glu Asp Gly Glu Lys Ala Ala Arg  
 20 25 30

&lt;210&gt; 127

&lt;211&gt; 18

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Nucleus target  
 sequence

&lt;400&gt; 127

agaaggaaac gacaaaag

18

&lt;210&gt; 128

<211> 6  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Nucleus target  
 sequence

<400> 128  
 Arg Arg Lys Arg Gln Lys  
 1 5

<210> 129  
 <211> 90  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Nucleolus  
 target sequence

<400> 129  
 agaaaacgta tacgtactta cctcaagtcc tgcaggcgga tgaaaagaag tggttttgag 60  
 atgtctcgac ctattccttc ccaccttact 90

<210> 130  
 <211> 30  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Nucleolus  
 target sequence

<400> 130  
 Arg Lys Arg Ile Arg Thr Tyr Leu Lys Ser Cys Arg Arg Met Lys Arg  
 1 5 10 15  
 Ser Gly Phe Glu Met Ser Arg Pro Ile Pro Ser His Leu Thr  
 20 25 30

<210> 131  
 <211> 87  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Mitochondria  
 target sequence

<400> 131  
 atgtccgctc tgacgccgct gctgctgcgg ggcttgacag gtcgggccg ggggtccca 60

gtgccgcgcgcg ccaagatcca ttcgttg

87

&lt;210&gt; 132

&lt;211&gt; 29

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Mitochondria  
target sequence

&lt;400&gt; 132

Met Ser Val Leu Thr Pro Leu Leu Leu Arg Gly Leu Thr Gly Ser Ala  
1 5 10 15

Arg Arg Leu Pro Val Pro Arg Ala Leu Ile His Ser Leu  
20 25

&lt;210&gt; 133

&lt;211&gt; 99

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Nuclear  
Envelope target sequence

&lt;400&gt; 133

atgagcattg ttttaataat tgttattgtg gtgatttttt taatatgttt tttatatatta 60

agcaacagca aagatcccag agtaccagtt gaattaatg

99

&lt;210&gt; 134

&lt;211&gt; 33

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Nuclear  
Envelope target sequence

&lt;400&gt; 134

Met Ser Ile Val Leu Ile Ile Val Ile Val Val Ile Phe Leu Ile Cys  
1 5 10 15

Phe Leu Tyr Leu Ser Asn Ser Lys Asp Pro Arg Val Pro Val Glu Leu  
20 25 30

Met

&lt;210&gt; 135

&lt;211&gt; 246

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Golgi target sequence

&lt;400&gt; 135

atgaggcttc gggagccgct cctgagcggc agcgccgcga tgccaggcgc gtccttacag 60  
 cgggcctgcc gcctgctcgt ggccgtctgc gctctgcacc ttggcgtcac cctcgtttac 120  
 tacctggctg gccgcgacct gagccgctg ccccaactgg tcggagtctc cacaccgctg 180  
 cagggcggct cgaacagtgc cgccgccatc gggcagtcct ccggggagct ccggaccgga 240  
 ggggcc 246

&lt;210&gt; 136

&lt;211&gt; 82

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Golgi target sequence

&lt;400&gt; 136

Met Arg Leu Arg Glu Pro Leu Leu Ser Gly Ser Ala Ala Met Pro Gly  
 1 5 10 15  
 Ala Ser Leu Gln Arg Ala Cys Arg Leu Leu Val Ala Val Cys Ala Leu  
 20 25 30  
 His Leu Gly Val Thr Leu Val Tyr Tyr Leu Ala Gly Arg Asp Leu Ser  
 35 40 45  
 Arg Leu Pro Gln Leu Val Gly Val Ser Thr Pro Leu Gln Gly Gly Ser  
 50 55 60  
 Asn Ser Ala Ala Ala Ile Gly Gln Ser Ser Gly Glu Leu Arg Thr Gly  
 65 70 75 80  
 Gly Ala

&lt;210&gt; 137

&lt;211&gt; 150

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Endoplasmic reticulum target sequence

<400> 137  
 gaaacaataa gacctataag aataagaaga tggtcttatt ttacatctac agacagcaaa 60  
 atggcaattc aattaagatc tccctttcca ttagcattac caggaatggt agctttatta 120  
 ggatggtggt gggtttttcag tagaaaaaaa 150

<210> 138  
 <211> 50  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Endoplasmic  
 reticulum target sequence

<400> 138  
 Glu Thr Ile Arg Pro Ile Arg Ile Arg Arg Cys Ser Tyr Phe Thr Ser  
 1 5 10 15  
 Thr Asp Ser Lys Met Ala Ile Gln Leu Arg Ser Pro Phe Pro Leu Ala  
 20 25 30  
 Leu Pro Gly Met Leu Ala Leu Leu Gly Trp Trp Trp Phe Phe Ser Arg  
 35 40 45  
 Lys Lys  
 50

<210> 139  
 <211> 39  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Nuclear Export  
 target sequence

<400> 139  
 gccttgacaga agaagctgga ggagctagag cttgatgag 39

<210> 140  
 <211> 13  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Nuclear Export  
 target sequence

<400> 140  
 Ala Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp Glu  
 1 5 10

<210> 141  
 <211> 1024  
 <212> DNA  
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Size exclusion  
 target sequence

<400> 141  
 gccgacctca gtcttggtga tgcgttgaca gaaccacctc cagaaattga gggagaaaata 60  
 aagcgagact tcatggctgc gctggaggca gagccctatg atgacatcgt gggagaaaact 120  
 gtggagaaaa ctgagtttat tcctctcctg gatggtgatg agaaaaccgg gaactcagag 180  
 tccaaaaaga aaccctgctt agacactagc caggttgaag gtatcccatc ttctaaacca 240  
 acactcctag ccaatggtga tcatggaatg gaggggaata aactgcagg gtctccaact 300  
 gacttccttg aagagagagt ggactatccg gattatcaga gcagccagaa ctggccagaa 360  
 gatgcaagct tttgtttcca gcctcagcaa gtgtagata ctgaccaggc tgagcccttt 420  
 aacgagcacc gtgatgatgg tttggcagat ctgctctttg tctccagtgg acccacgaac 480  
 gcttctgcat ttacagagcg agacaatcct tcagaagaca gttacggtat gcttccctgt 540  
 gactcatttg cttccacggc tgttgatatc caggagtggc ctgtgggagc cccaaactct 600  
 ccatgttcag agtcctgtgt ctcccagag gttactatag aaacctaca gccagcaaca 660  
 gagctctcca aggcagcaga agtggaatca gtgaaagagc agctgccagc taaagcattg 720  
 gaaacgatgg cagagcagac cactgatgtg gtgcactctc catccacaga cacaacacca 780  
 ggcccagaca cagaggcagc actggctaaa gacatagaag agatcaccaa gccagatgtg 840  
 atattggcaa atgtcacgca gccatctact gaatcggata tgttcctggc ccaggacatg 900  
 gaactactca caggaacaga ggcagcccac gctaacaata tcatattgcc tacagaacca 960  
 gacgaatctt caaccaagga tgtagacca cctatggaag aagaaattgt ccagggcaat 1020  
 gata 1024

<210> 142  
 <211> 566  
 <212> PRT  
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Size exclusion  
 target sequence

<400> 142  
 Ala Asp Leu Ser Leu Val Asp Ala Leu Thr Glu Pro Pro Pro Glu Ile  
 1 5 10 15  
 Glu Gly Glu Ile Lys Arg Asp Phe Met Ala Ala Leu Glu Ala Glu Pro  
 20 25 30  
 Tyr Asp Asp Ile Val Gly Glu Thr Val Glu Lys Thr Glu Phe Ile Pro  
 35 40 45  
 Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys Lys  
 50 55 60  
 Pro Cys Leu Asp Thr Ser Gln Val Glu Gly Ile Pro Ser Ser Lys Pro  
 65 70 75 80  
 Thr Leu Leu Ala Asn Gly Asp His Gly Met Glu Gly Asn Asn Thr Ala  
 85 90 95  
 Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp Tyr  
 100 105 110  
 Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln Pro  
 115 120 125  
 Gln Gln Val Leu Asp Thr Asp Gln Ala Glu Pro Phe Asn Glu His Arg  
 130 135 140  
 Asp Asp Gly Leu Ala Asp Leu Leu Phe Val Ser Ser Gly Pro Thr Asn  
 145 150 155 160  
 Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr Gly  
 165 170 175  
 Met Leu Pro Cys Asp Ser Phe Ala Ser Thr Ala Val Val Ser Gln Glu  
 180 185 190  
 Trp Ser Val Gly Ala Pro Asn Ser Pro Cys Ser Glu Ser Cys Val Ser  
 195 200 205  
 Pro Glu Val Thr Ile Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser Lys  
 210 215 220  
 Ala Ala Glu Val Glu Ser Val Lys Glu Gln Leu Pro Ala Lys Ala Leu  
 225 230 235 240  
 Glu Thr Met Ala Glu Gln Thr Thr Asp Val Val His Ser Pro Ser Thr  
 245 250 255  
 Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp Ile  
 260 265 270  
 Glu Glu Ile Thr Lys Pro Asp Val Ile Leu Ala Asn Val Thr Gln Pro  
 275 280 285  
 Ser Thr Glu Ser Asp Met Phe Leu Ala Gln Asp Met Glu Leu Leu Thr  
 290 295 300

Gly Thr Glu Ala Ala His Ala Asn Asn Ile Ile Leu Pro Thr Glu Pro  
 305 310 315 320  
 Asp Glu Ser Ser Thr Lys Asp Val Ala Pro Pro Met Glu Glu Glu Ile  
 325 330 335  
 Val Pro Gly Asn Asp Thr Thr Ser Pro Lys Glu Thr Glu Thr Thr Leu  
 340 345 350  
 Pro Ile Lys Met Asp Leu Ala Pro Pro Glu Asp Val Leu Leu Thr Lys  
 355 360 365  
 Glu Thr Glu Leu Ala Pro Ala Lys Gly Met Val Ser Leu Ser Glu Ile  
 370 375 380  
 Glu Glu Ala Leu Ala Lys Asn Asp Val Arg Ser Ala Glu Ile Pro Val  
 385 390 395 400  
 Ala Gln Glu Thr Val Val Ser Glu Thr Glu Val Val Leu Ala Thr Glu  
 405 410 415  
 Val Val Leu Pro Ser Asp Pro Ile Thr Thr Leu Thr Lys Asp Val Thr  
 420 425 430  
 Leu Pro Leu Glu Ala Glu Arg Pro Leu Val Thr Asp Met Thr Pro Ser  
 435 440 445  
 Leu Glu Thr Glu Met Thr Leu Gly Lys Glu Thr Ala Pro Pro Thr Glu  
 450 455 460  
 Thr Asn Leu Gly Met Ala Lys Asp Met Ser Pro Leu Pro Glu Ser Glu  
 465 470 475 480  
 Val Thr Leu Gly Lys Asp Val Val Ile Leu Pro Glu Thr Lys Val Ala  
 485 490 495  
 Glu Phe Asn Asn Val Thr Pro Leu Ser Glu Glu Glu Val Thr Ser Val  
 500 505 510  
 Lys Asp Met Ser Pro Ser Ala Glu Thr Glu Ala Pro Leu Ala Lys Asn  
 515 520 525  
 Ala Asp Leu His Ser Gly Thr Glu Leu Ile Val Asp Asn Ser Met Ala  
 530 535 540  
 Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu Thr Lys Val Ala Thr Val  
 545 550 555 560  
 Pro Ile Lys Asp Lys Gly  
 565

&lt;210&gt; 143

&lt;211&gt; 63

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence



&lt;220&gt;

<223> Description of Artificial Sequence: Vesicle  
membrane target sequence

&lt;400&gt; 143

atgtgggcaa tcgggattac tgttctgggt atcttcatca tcatcatcat cgtgtgggtt 60  
gtc 63

&lt;210&gt; 144

&lt;211&gt; 21

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Vesicle  
membrane target sequence

&lt;400&gt; 144

Met Trp Ala Ile Gly Ile Thr Val Leu Val Ile Phe Ile Ile Ile Ile  
1 5 10 15  
Ile Val Trp Val Val  
20

&lt;210&gt; 145

&lt;211&gt; 61

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Vesicle  
membrane target sequence

&lt;400&gt; 145

atgtgggcga tagggatcag tgtcctgggtg atcattgtca tcatcatcat cgtgtgggtg 60  
g 61

&lt;210&gt; 146

&lt;211&gt; 20

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Vesicle  
membrane target sequence

&lt;400&gt; 146

Met Trp Ala Ile Gly Ile Ser Val Leu Val Ile Ile Val Ile Ile Ile  
1 5 10 15  
Ile Val Trp Cys

20

<210> 147  
<211> 39  
<212> DNA  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Nuclear Export  
target sequence

&lt;400&gt; 147

gacctgcaga agaagctgga ggagctggaa cttgacgag

39

<210> 148  
<211> 13  
<212> PRT  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Nuclear Export  
target sequence

&lt;400&gt; 148

Asp Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp Glu  
1 5 10

<210> 149  
<211> 9  
<212> DNA  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Peroxisome  
target sequence

&lt;400&gt; 149

tctaaactg

9

<210> 150  
<211> 3  
<212> PRT  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Peroxisome  
target sequence

&lt;400&gt; 150

Ser Lys Leu  
1

<210> 151  
 <211> 3378  
 <212> DNA  
 <213> Mus musculus

<220>  
 <221> CDS  
 <222> (1)..(3375)

<400> 151  
 atg gcc gac ctc agt ctt gtg gat gcg ttg aca gaa cca cct cca gaa 48  
 Met Ala Asp Leu Ser Leu Val Asp Ala Leu Thr Glu Pro Pro Pro Glu  
 1 5 10 15  
 att gag gga gaa ata aag cga gac ttc atg gct gcg ctg gag gca gag 96  
 Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala Ala Leu Glu Ala Glu  
 20 25 30  
 ccc tat gat gac atc gtg gga gaa act gtg gag aaa act gag ttt att 144  
 Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu Lys Thr Glu Phe Ile  
 35 40 45  
 cct ctc ctg gat ggt gat gag aaa acc ggg aac tca gag tcc aaa aag 192  
 Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys  
 50 55 60  
 aaa ccc tgc tta gac act agc cag gtt gaa ggt atc cca tct tct aaa 240  
 Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly Ile Pro Ser Ser Lys  
 65 70 75 80  
 cca aca ctc cta gcc aat ggt gat cat gga atg gag ggg aat aac act 288  
 Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met Glu Gly Asn Asn Thr  
 85 90 95  
 gca ggg tct cca act gac ttc ctt gaa gag aga gtg gac tat ccg gat 336  
 Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp  
 100 105 110  
 tat cag agc agc cag aac tgg cca gaa gat gca agc ttt tgt ttc cag 384  
 Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln  
 115 120 125  
 cct cag caa gtg tta gat act gac cag gct gag ccc ttt aac gag cac 432  
 Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu Pro Phe Asn Glu His  
 130 135 140  
 cgt gat gat ggt ttg gca gat ctg ctc ttt gtc tcc agt gga ccc acg 480  
 Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val Ser Ser Gly Pro Thr  
 145 150 155  
 aac gct tct gca ccc gaa gaa gac aat cct tca gaa gac agt tac 528  
 Asn Ala Ser Ala Phe Thr Glu Arg Thr Pro Ser Glu Asp Ser Tyr  
 165 170 175  
 ggt atg ctt ccc tgt gac tca ttt gct tcc acg gct gtt gta tct cag  
 Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr Ala Val Val Ser Gln  
 180 185 190

gag tgg tct gtg gga gcc cca aac tct cca tgt tca gag tcc tgt gtc	624
Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys Ser Glu Ser Cys Val	
195 200 205	
tcc cca gag gtt act ata gaa acc cta cag cca gca aca gag ctc tcc	672
Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser	
210 215 220	
aag gca gca gaa gtg gaa tca gtg aaa gag cag ctg cca gct aaa gca	720
Lys Ala Ala Glu Val Glu Ser Val Lys Glu Gln Leu Pro Ala Lys Ala	
225 230 235 240	
ttg gaa acg atg gca gag cag acc act gat gtg gtg cac tct cca tcc	768
Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val Val His Ser Pro Ser	
245 250 255	
aca gac aca aca cca ggc cca gac aca gag gca gca ctg gct aaa gac	816
Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp	
260 265 270	
ata gaa gag atc acc aag cca gat gtg ata ttg gca aat gtc acg cag	864
Ile Glu Glu Ile Thr Lys Pro Asp Val Ile Leu Ala Asn Val Thr Gln	
275 280 285	
cca tct act gaa tcg gat atg ttc ctg gcc cag gac atg gaa cta ctc	912
Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln Asp Met Glu Leu Leu	
290 295 300	
aca gga aca gag gca gcc cac gct aac aat atc ata ttg cct aca gaa	960
Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile Ile Leu Pro Thr Glu	
305 310 315 320	
cca gac gaa tct tca acc aag gat gta gca cca cct atg gaa gaa gaa	1008
Pro Asp Glu Ser Ser Thr Lys Asp Val Ala Pro Pro Met Glu Glu Glu	
325 330 335	
att gtc cca ggc aat gat acg aca tcc ccc aaa gaa aca gag aca aca	1056
Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys Glu Thr Glu Thr Thr	
340 345 350	
ctt cca ata aaa atg gac ttg gca cca cct gag gat gtg tta ctt acc	1104
Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu Asp Val Leu Leu Thr	
355 360 365	
aaa gaa aca gaa cta gcc cca gcc aag ggc atg gtt tca ctc tca gaa	1152
Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met Val Ser Leu Ser Glu	
370 375 380	
ata gaa gag gct ctg gca aag aat gat gtt cgc tct gca gaa ata cct	1200
Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg Ser Ala Glu Ile Pro	
385 390 395 400	
gtg gct cag gag aca gtg gtc tca gaa aca gag gtg gtc ctg gca aca	1248
Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu Val Val Leu Ala Thr	
405 410 415	

gaa	gtg	gta	ctg	ccc	tca	gat	ccc	ata	aca	aca	ttg	aca	aag	gat	gtg	1296
Glu	Val	Val	Leu	Pro	Ser	Asp	Pro	Ile	Thr	Thr	Leu	Thr	Lys	Asp	Val	
			420					425					430			
aca	ctc	ccc	tta	gaa	gca	gag	aga	ccg	ttg	gtg	acg	gac	atg	act	cca	1344
Thr	Leu	Pro	Leu	Glu	Ala	Glu	Arg	Pro	Leu	Val	Thr	Asp	Met	Thr	Pro	
			435				440					445				
tct	ctg	gaa	aca	gaa	atg	acc	cta	ggc	aaa	gag	aca	gct	cca	ccc	aca	1392
Ser	Leu	Glu	Thr	Glu	Met	Thr	Leu	Gly	Lys	Glu	Thr	Ala	Pro	Pro	Thr	
			450				455				460					
gaa	aca	aat	ttg	ggc	atg	gcc	aaa	gac	atg	tct	cca	ctc	cca	gaa	tca	1440
Glu	Thr	Asn	Leu	Gly	Met	Ala	Lys	Asp	Met	Ser	Pro	Leu	Pro	Glu	Ser	
465					470					475					480	
gaa	gtg	act	ctg	ggc	aag	gac	gtg	gtt	ata	ctt	cca	gaa	aca	aag	gtg	1488
Glu	Val	Thr	Leu	Gly	Lys	Asp	Val	Val	Ile	Leu	Pro	Glu	Thr	Lys	Val	
				485					490					495		
gct	gag	ttt	aac	aat	gtg	act	cca	ctt	tca	gaa	gaa	gag	gta	acc	tca	1536
Ala	Glu	Phe	Asn	Asn	Val	Thr	Pro	Leu	Ser	Glu	Glu	Glu	Val	Thr	Ser	
			500					505					510			
gtc	aag	gac	atg	tct	ccg	tct	gca	gaa	aca	gag	gct	ccc	ctg	gct	aag	1584
Val	Lys	Asp	Met	Ser	Pro	Ser	Ala	Glu	Thr	Glu	Ala	Pro	Leu	Ala	Lys	
			515				520					525				
aat	gct	gat	ctg	cac	tca	gga	aca	gag	ctg	att	gtg	gac	aac	agc	atg	1632
Asn	Ala	Asp	Leu	His	Ser	Gly	Thr	Glu	Leu	Ile	Val	Asp	Asn	Ser	Met	
			530				535				540					
gct	cca	gcc	tcc	gat	ctt	gca	ctg	ccc	ttg	gaa	aca	aaa	gta	gca	aca	1680
Ala	Pro	Ala	Ser	Asp	Leu	Ala	Leu	Pro	Leu	Glu	Thr	Lys	Val	Ala	Thr	
545						550				555					560	
gtt	cca	att	aaa	gac	aaa	gga	act	gta	cag	act	gaa	gaa	aaa	cca	cgt	1728
Val	Pro	Ile	Lys	Asp	Lys	Gly	Thr	Val	Gln	Thr	Glu	Glu	Lys	Pro	Arg	
				565				570						575		
gaa	gac	tcc	cag	tta	gca	tct	atg	cag	cac	aag	gga	cag	tca	aca	gta	1776
Glu	Asp	Ser	Gln	Leu	Ala	Ser	Met	Gln	His	Lys	Gly	Gln	Ser	Thr	Val	
			580					585					590			
cct	cct	tgc	acg	gct	tca	cca	gaa	cca	gtc	aaa	gct	gca	gaa	caa	atg	1824
Pro	Pro	Cys	Thr	Ala	Ser	Pro	Glu	Pro	Val	Lys	Ala	Ala	Glu	Gln	Met	
			595				600					605				
tct	acc	tta	cca	ata	gat	gca	cct	tct	cca	tta	gag	aac	tta	gag	cag	1872
Ser	Thr	Leu	Pro	Ile	Asp	Ala	Pro	Ser	Pro	Leu	Glu	Asn	Leu	Glu	Gln	
			610				615				620					
aag	gaa	acg	cct	ggc	agc	cag	cct	tct	gag	cct	tgc	tca	gga	gta	tcc	1920
Lys	Glu	Thr	Pro	Gly	Ser	Gln	Pro	Ser	Glu	Pro	Cys	Ser	Gly	Val	Ser	
625					630					635					640	
cgg	caa	gaa	gaa	gca	aag	gct	gct	gta	ggg	gtg	act	gga	aat	gac	atc	1968

Arg	Gln	Glu	Glu	Ala	Lys	Ala	Ala	Val	Gly	Val	Thr	Gly	Asn	Asp	Ile	
				645					650					655		
act	acc	ccg	cca	aac	aag	gag	cca	cca	cca	agc	cca	gaa	aag	aaa	gca	2016
Thr	Thr	Pro	Pro	Asn	Lys	Glu	Pro	Pro	Pro	Ser	Pro	Glu	Lys	Lys	Ala	
			660					665					670			
aag	cct	ttg	gcc	acc	act	caa	cct	gca	aag	act	tca	aca	tcg	aaa	gcc	2064
Lys	Pro	Leu	Ala	Thr	Thr	Gln	Pro	Ala	Lys	Thr	Ser	Thr	Ser	Lys	Ala	
		675					680					685				
aaa	aca	cag	ccc	act	tct	ctc	cct	aag	caa	cca	gct	ccc	acc	acc	tct	2112
Lys	Thr	Gln	Pro	Thr	Ser	Leu	Pro	Lys	Gln	Pro	Ala	Pro	Thr	Thr	Ser	
		690				695					700					
ggg	ggg	ttg	aat	aaa	aaa	ccc	atg	agc	ctc	gcc	tca	ggc	tca	gtg	cca	2160
Gly	Gly	Leu	Asn	Lys	Lys	Pro	Met	Ser	Leu	Ala	Ser	Gly	Ser	Val	Pro	
705				710					715						720	
gct	gcc	cca	cac	aaa	cgc	cct	gct	gct	gcc	act	gct	act	gcc	agg	cct	2208
Ala	Ala	Pro	His	Lys	Arg	Pro	Ala	Ala	Ala	Thr	Ala	Thr	Ala	Arg	Pro	
				725					730					735		
tcc	acc	cta	cct	gcc	aga	gac	gtg	aag	cca	aag	cca	att	aca	gaa	gct	2256
Ser	Thr	Leu	Pro	Ala	Arg	Asp	Val	Lys	Pro	Lys	Pro	Ile	Thr	Glu	Ala	
			740					745					750			
aag	gtt	gcc	gaa	aag	cgg	acc	tct	cca	tcc	aag	cct	tca	tct	gcc	cca	2304
Lys	Val	Ala	Glu	Lys	Arg	Thr	Ser	Pro	Ser	Lys	Pro	Ser	Ser	Ala	Pro	
		755					760					765				
gcc	ctc	aaa	cct	gga	cct	aaa	acc	acc	cca	acc	gtt	tca	aaa	gcc	aca	2352
Ala	Leu	Lys	Pro	Gly	Pro	Lys	Thr	Thr	Pro	Thr	Val	Ser	Lys	Ala	Thr	
		770				775					780					
tct	ccc	tca	act	ctt	gtt	tcc	act	gga	cca	agt	agt	aga	agt	cca	gct	2400
Ser	Pro	Ser	Thr	Leu	Val	Ser	Thr	Gly	Pro	Ser	Ser	Arg	Ser	Pro	Ala	
785					790					795					800	
aca	act	ctg	cct	aag	agg	cca	acc	agc	atc	aag	act	gag	ggg	aaa	cct	2448
Thr	Thr	Leu	Pro	Lys	Arg	Pro	Thr	Ser	Ile	Lys	Thr	Glu	Gly	Lys	Pro	
				805					810					815		
gct	gat	gtc	aaa	agg	atg	act	gct	aag	tct	gcc	tca	gct	gac	ttg	agt	2496
Ala	Asp	Val	Lys	Arg	Met	Thr	Ala	Lys	Ser	Ala	Ser	Ala	Asp	Leu	Ser	
			820					825					830			
cgc	tca	aag	acc	acc	tct	gcc	agt	tct	gtg	aag	aga	aac	acc	act	ccc	2544
Arg	Ser	Lys	Thr	Thr	Ser	Ala	Ser	Ser	Val	Lys	Arg	Asn	Thr	Thr	Pro	
		835					840					845				
act	ggg	gca	gca	ccc	cca	gca	ggg	atg	act	tcc	act	cga	gtc	aag	ccc	2592
Thr	Gly	Ala	Ala	Pro	Pro	Ala	Gly	Met	Thr	Ser	Thr	Arg	Val	Lys	Pro	
		850				855					860					
atg	tct	gca	cct	agc	cgc	tct	tct	ggg	gct	ctt	tct	gtg	gac	aag	aag	2640
Met	Ser	Ala	Pro	Ser	Arg	Ser	Ser	Gly	Ala	Leu	Ser	Val	Asp	Lys	Lys	

865	870	875	880	
ccc act tcc act aag cct agc tcc tct gct ccc agg gtg agc cgc ctg				2688
Pro Thr Ser Thr Lys Pro Ser Ser Ser Ala Pro Arg Val Ser Arg Leu				
885		890	895	
gcc aca act gtt tct gcc cct gac ctg aag agt gtt cgc tcc aag gtc				2736
Ala Thr Thr Val Ser Ala Pro Asp Leu Lys Ser Val Arg Ser Lys Val				
900		905	910	
ggc tct aca gaa aac atc aaa cac cag cct gga gga ggc cgg gcc aaa				2784
Gly Ser Thr Glu Asn Ile Lys His Gln Pro Gly Gly Gly Arg Ala Lys				
915		920	925	
gta gag aaa aaa aca gag gca gct acc aca gct ggg aag cct gaa cct				2832
Val Glu Lys Lys Thr Glu Ala Ala Thr Thr Ala Gly Lys Pro Glu Pro				
930	935		940	
aat gca gtc act aaa gca gcc ggc tcc att gcg agt gca cag aaa ccg				2880
Asn Ala Val Thr Lys Ala Ala Gly Ser Ile Ala Ser Ala Gln Lys Pro				
945	950		955	960
cct gct ggg aaa gtc cag ata gta tcc aaa aaa gtg agc tac agt cat				2928
Pro Ala Gly Lys Val Gln Ile Val Ser Lys Lys Val Ser Tyr Ser His				
965		970		975
att caa tcc aag tgt gtt tcc aag gac aat att aag cat gtc cct gga				2976
Ile Gln Ser Lys Cys Val Ser Lys Asp Asn Ile Lys His Val Pro Gly				
980		985		990
tgt ggc aat gtt cag att cag aac aag aaa gtg gac ata tcc aag gtc				3024
Cys Gly Asn Val Gln Ile Gln Asn Lys Lys Val Asp Ile Ser Lys Val				
995	1000		1005	
tcc tcc aag tgt ggg tcc aaa gct aat atc aag cac aag cct ggt gga				3072
Ser Ser Lys Cys Gly Ser Lys Ala Asn Ile Lys His Lys Pro Gly Gly				
1010	1015		1020	
gga gat gtc aag att gaa agt cag aag ttg aac ttc aag gag aag gcc				3120
Gly Asp Val Lys Ile Glu Ser Gln Lys Leu Asn Phe Lys Glu Lys Ala				
1025	1030		1035	1040
caa gcc aaa gtg gga tcc ctt gat aac gtt ggc cac ttt cct gca gga				3168
Gln Ala Lys Val Gly Ser Leu Asp Asn Val Gly His Phe Pro Ala Gly				
1045		1050		1055
ggt gcc gtg aag act gag ggc ggt ggc agt gag gcc ctt ccg tgt cca				3216
Gly Ala Val Lys Thr Glu Gly Gly Ser Glu Ala Leu Pro Cys Pro				
1060		1065		1070
ggc ccc ccc gct ggg gag gag cca gtc atc cct gag gct gcg cct gac				3264
Gly Pro Pro Ala Gly Glu Glu Pro Val Ile Pro Glu Ala Ala Pro Asp				
1075		1080		1085
cgt ggc gcc cct act tca gcc agt ggc ctc agt ggc cac acc acc ctg				3312
Arg Gly Ala Pro Thr Ser Ala Ser Gly Leu Ser Gly His Thr Thr Leu				
1090	1095		1100	

tca ggg ggt ggt gac caa agg gag ccc cag acc ttg gac agc cag atc 3360  
 Ser Gly Gly Gly Asp Gln Arg Glu Pro Gln Thr Leu Asp Ser Gln Ile  
 1105 1110 1115 1120

cag gag aca agc atc taa 3378  
 Gln Glu Thr Ser Ile  
 1125

<210> 152

<211> 1125

<212> PRT

<213> Mus musculus

<400> 152

Met Ala Asp Leu Ser Leu Val Asp Ala Leu Thr Glu Pro Pro Pro Glu  
 1 5 10 15

Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala Ala Leu Glu Ala Glu  
 20 25 30

Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu Lys Thr Glu Phe Ile  
 35 40 45

Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys  
 50 55 60

Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly Ile Pro Ser Ser Lys  
 65 70 75 80

Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met Glu Gly Asn Asn Thr  
 85 90 95

Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp  
 100 105 110

Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln  
 115 120 125

Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu Pro Phe Asn Glu His  
 130 135 140

Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val Ser Ser Gly Pro Thr  
 145 150 155 160

Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr  
 165 170 175

Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr Ala Val Val Ser Gln  
 180 185 190

Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys Ser Glu Ser Cys Val  
 195 200 205

Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser  
 210 215 220



Lys Ala Ala Glu Val Glu Ser Val Lys Glu Gln Leu Pro Ala Lys Ala  
 225 230 235 240  
 Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val Val His Ser Pro Ser  
 245 250 255  
 Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp  
 260 265 270  
 Ile Glu Glu Ile Thr Lys Pro Asp Val Ile Leu Ala Asn Val Thr Gln  
 275 280 285  
 Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln Asp Met Glu Leu Leu  
 290 295 300  
 Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile Ile Leu Pro Thr Glu  
 305 310 315 320  
 Pro Asp Glu Ser Ser Thr Lys Asp Val Ala Pro Pro Met Glu Glu Glu  
 325 330 335  
 Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys Glu Thr Glu Thr Thr  
 340 345 350  
 Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu Asp Val Leu Leu Thr  
 355 360 365  
 Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met Val Ser Leu Ser Glu  
 370 375 380  
 Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg Ser Ala Glu Ile Pro  
 385 390 395 400  
 Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu Val Val Leu Ala Thr  
 405 410 415  
 Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr Leu Thr Lys Asp Val  
 420 425 430  
 Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val Thr Asp Met Thr Pro  
 435 440 445  
 Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu Thr Ala Pro Pro Thr  
 450 455 460  
 Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser Pro Leu Pro Glu Ser  
 465 470 475 480  
 Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu Pro Glu Thr Lys Val  
 485 490 495  
 Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu Glu Glu Val Thr Ser  
 500 505 510  
 Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu Ala Pro Leu Ala Lys  
 515 520 525

Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile Val Asp Asn Ser Met  
 530 535 540  
 Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu Thr Lys Val Ala Thr  
 545 550 555 560  
 Val Pro Ile Lys Asp Lys Gly Thr Val Gln Thr Glu Glu Lys Pro Arg  
 565 570 575  
 Glu Asp Ser Gln Leu Ala Ser Met Gln His Lys Gly Gln Ser Thr Val  
 580 585 590  
 Pro Pro Cys Thr Ala Ser Pro Glu Pro Val Lys Ala Ala Glu Gln Met  
 595 600 605  
 Ser Thr Leu Pro Ile Asp Ala Pro Ser Pro Leu Glu Asn Leu Glu Gln  
 610 615 620  
 Lys Glu Thr Pro Gly Ser Gln Pro Ser Glu Pro Cys Ser Gly Val Ser  
 625 630 635 640  
 Arg Gln Glu Glu Ala Lys Ala Ala Val Gly Val Thr Gly Asn Asp Ile  
 645 650 655  
 Thr Thr Pro Pro Asn Lys Glu Pro Pro Pro Ser Pro Glu Lys Lys Ala  
 660 665 670  
 Lys Pro Leu Ala Thr Thr Gln Pro Ala Lys Thr Ser Thr Ser Lys Ala  
 675 680 685  
 Lys Thr Gln Pro Thr Ser Leu Pro Lys Gln Pro Ala Pro Thr Thr Ser  
 690 695 700  
 Gly Gly Leu Asn Lys Lys Pro Met Ser Leu Ala Ser Gly Ser Val Pro  
 705 710 715 720  
 Ala Ala Pro His Lys Arg Pro Ala Ala Ala Thr Ala Thr Ala Arg Pro  
 725 730 735  
 Ser Thr Leu Pro Ala Arg Asp Val Lys Pro Lys Pro Ile Thr Glu Ala  
 740 745 750  
 Lys Val Ala Glu Lys Arg Thr Ser Pro Ser Lys Pro Ser Ser Ala Pro  
 755 760 765  
 Ala Leu Lys Pro Gly Pro Lys Thr Thr Pro Thr Val Ser Lys Ala Thr  
 770 775 780  
 Ser Pro Ser Thr Leu Val Ser Thr Gly Pro Ser Ser Arg Ser Pro Ala  
 785 790 795 800  
 Thr Thr Leu Pro Lys Arg Pro Thr Ser Ile Lys Thr Glu Gly Lys Pro  
 805 810 815  
 Ala Asp Val Lys Arg Met Thr Ala Lys Ser Ala Ser Ala Asp Leu Ser  
 820 825 830

Arg Ser Lys Thr Thr Ser Ala Ser Ser Val Lys Arg Asn Thr Thr Pro  
 835 840 845  
 Thr Gly Ala Ala Pro Pro Ala Gly Met Thr Ser Thr Arg Val Lys Pro  
 850 855 860  
 Met Ser Ala Pro Ser Arg Ser Ser Gly Ala Leu Ser Val Asp Lys Lys  
 865 870 875 880  
 Pro Thr Ser Thr Lys Pro Ser Ser Ser Ala Pro Arg Val Ser Arg Leu  
 885 890 895  
 Ala Thr Thr Val Ser Ala Pro Asp Leu Lys Ser Val Arg Ser Lys Val  
 900 905 910  
 Gly Ser Thr Glu Asn Ile Lys His Gln Pro Gly Gly Gly Arg Ala Lys  
 915 920 925  
 Val Glu Lys Lys Thr Glu Ala Ala Thr Thr Ala Gly Lys Pro Glu Pro  
 930 935 940  
 Asn Ala Val Thr Lys Ala Ala Gly Ser Ile Ala Ser Ala Gln Lys Pro  
 945 950 955 960  
 Pro Ala Gly Lys Val Gln Ile Val Ser Lys Lys Val Ser Tyr Ser His  
 965 970 975  
 Ile Gln Ser Lys Cys Val Ser Lys Asp Asn Ile Lys His Val Pro Gly  
 980 985 990  
 Cys Gly Asn Val Gln Ile Gln Asn Lys Lys Val Asp Ile Ser Lys Val  
 995 1000 1005  
 Ser Ser Lys Cys Gly Ser Lys Ala Asn Ile Lys His Lys Pro Gly Gly  
 1010 1015 1020  
 Gly Asp Val Lys Ile Glu Ser Gln Lys Leu Asn Phe Lys Glu Lys Ala  
 1025 1030 1035 1040  
 Gln Ala Lys Val Gly Ser Leu Asp Asn Val Gly His Phe Pro Ala Gly  
 1045 1050 1055  
 Gly Ala Val Lys Thr Glu Gly Gly Gly Ser Glu Ala Leu Pro Cys Pro  
 1060 1065 1070  
 Gly Pro Pro Ala Gly Glu Glu Pro Val Ile Pro Glu Ala Ala Pro Asp  
 1075 1080 1085  
 Arg Gly Ala Pro Thr Ser Ala Ser Gly Leu Ser Gly His Thr Thr Leu  
 1090 1095 1100  
 Ser Gly Gly Gly Asp Gln Arg Glu Pro Gln Thr Leu Asp Ser Gln Ile  
 1105 1110 1115 1120  
 Gln Glu Thr Ser Ile  
 1125

<210> 153  
<211> 96  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 153  
tcacatcatccg gagctggagc cggagctggc cgcacggctg ttaaactctga aggaaagaga 60  
aagtgtgacg aagttgatgg aattgatgaa gtagca 96

<210> 154  
<211> 99  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 154  
gaagaaggat ccggcacttg ggggtgtaga atgaacaccc tccaagctga gcttgacacag 60  
gatttcgtgg acagtagaca tagtacttgc tacttcac 99

<210> 155  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 155  
tcacatcatccg gagctgga 18

<210> 156  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 156  
gaagaaggat ccggcact 18

<210> 157  
<211> 96  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide

<400> 157  
tcacatcatccg gaagaaggaa acgacaaaag cgatcggctg tttaatctga aggaaagaga 60  
aagtgtgacg aagttgatgg aattgatgaa gtagca 96

<210> 158  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide

<400> 158  
tcacatcatccg gaagaagg 18

<210> 159  
<211> 60  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide

<400> 159  
tcacatcatccg gaagaaggaa acgacaaaag cgatcgacaa gacttggtga aattgacaac 60

<210> 160  
<211> 99  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide

<400> 160  
gaagaaggat ccggcacttg ggggtgtaga atgaacaccc tocaagctga gcttgacacag 60  
gatttcgtgg acagtagaca tagtactgtt gtcaatttc 99

<210> 161  
<211> 84  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 161  
tcatcatccg gaagaaggaa acgacaaaag cgatcgtatc aaaaaggaa accagttgaa 60  
acagacagcg aagagcaacc ttat 84

<210> 162  
<211> 99  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 162  
gaagaaggat ccggcacttg ggggtgtaga atgaacaccc tccaagctga gcttgcacag 60  
gatttcgtgg acagtagaca tagtactata aggttgctc 99

<210> 163  
<211> 60  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 163  
tcatcatccg gaagaaaacg tatacgtact tacctcaagt cctgcaggcg gatgaaaaga 60

<210> 164  
<211> 63  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 164  
gaagaacgat cgagtaaggt gggaaggaat aggtcgagac atctcaaaac cacttctttt 60  
cat 63

<210> 165  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 165  
tcatcatccg gaagaaaa

18

<210> 166  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
oligonucleotide

<400> 166  
gaagaacgat cgagtaag

18

<210> 167  
<211> 14  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-1,4,5  
substrate recognition sequence

<400> 167  
ttagaacatg acaa

14

<210> 168  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Caspase-1,4,5  
substrate recognition sequence

<400> 168  
Leu Glu His Asp  
1

<210> 169  
<211> 1380  
<212> DNA  
<213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: GFP-HSP27

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(1380)

&lt;400&gt; 169

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg	48
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag	240
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag	288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	



ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	
225 230 235 240	
gga ctc aga tct cga gcg gcg tcc aga gca gag tca gcc agc atg acc	768
Gly Leu Arg Ser Arg Ala Ala Ser Arg Ala Glu Ser Ala Ser Met Thr	
245 250 255	
gag cgc cgc gtc ccc ttc tcg ctc ctg cgg ggc ccc agc tgg gac ccc	816
Glu Arg Arg Val Pro Phe Ser Leu Leu Arg Gly Pro Ser Trp Asp Pro	
260 265 270	
ttc cgc gac tgg tac ccg cat agc cgc ctc ttc gac cag gcc ttc ggg	864
Phe Arg Asp Trp Tyr Pro His Ser Arg Leu Phe Asp Gln Ala Phe Gly	
275 280 285	
ctg ccc cgg ctg ccg gag gag tgg tcg cag tgg tta ggc ggc agc agc	912
Leu Pro Arg Leu Pro Glu Glu Trp Ser Gln Trp Leu Gly Gly Ser Ser	
290 295 300	
tgg cca ggc tac gtg cgc ccc ctg ccc ccc gcc gcc atc gag agc ccc	960
Trp Pro Gly Tyr Val Arg Pro Leu Pro Pro Ala Ala Ile Glu Ser Pro	
305 310 315 320	
gca gtg gcc gcg ccc gcc tac agc cgc gcg ctc agc cgg caa ctc agc	1008
Ala Val Ala Ala Pro Ala Tyr Ser Arg Ala Leu Ser Arg Gln Leu Ser	
325 330 335	
agc ggg gtc tcg gag atc cgg cac act gcg gac cgc tgg cgc gtg tcc	1056
Ser Gly Val Ser Glu Ile Arg His Thr Ala Asp Arg Trp Arg Val Ser	
340 345 350	
ctg gat gtc aac cac ttc gcc ccg gac gag ctg acg gtc aag acc aag	1104
Leu Asp Val Asn His Phe Ala Pro Asp Glu Leu Thr Val Lys Thr Lys	
355 360 365	
gat ggc gtg gtg gag atc acc ggc aag cac gag gag cgg cag gac gag	1152
Asp Gly Val Val Glu Ile Thr Gly Lys His Glu Glu Arg Gln Asp Glu	
370 375 380	
cat ggc tac atc tcc cgg tgc ttc acg cgg aaa tac acg ctg ccc ccc	1200
His Gly Tyr Ile Ser Arg Cys Phe Thr Arg Lys Tyr Thr Leu Pro Pro	
385 390 395 400	
ggt gtg gac ccc acc caa gtt tcc tcc tcc ctg tcc cct gag ggc aca	1248
Gly Val Asp Pro Thr Gln Val Ser Ser Ser Leu Ser Pro Glu Gly Thr	
405 410 415	
ctg acc gtg gag gcc ccc atg ccc aag cta gcc acg cag tcc aac gag	1296

Leu Thr Val Glu Ala Pro Met Pro Lys Leu Ala Thr Gln Ser Asn Glu  
 420 425 430

atc acc atc cca gtc acc ttc gag tcg cgg gcc cag ctt ggg ggc cca 1344  
 Ile Thr Ile Pro Val Thr Phe Glu Ser Arg Ala Gln Leu Gly Gly Pro  
 435 440 445

gaa gct gca aaa tcc gat gag act gcc gcc aag taa 1380  
 Glu Ala Ala Lys Ser Asp Glu Thr Ala Ala Lys  
 450 455 460

<210> 170  
 <211> 459  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: GFP-HSP27

<400> 170  
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240  
 Gly Leu Arg Ser Arg Ala Ala Ser Arg Ala Glu Ser Ala Ser Met Thr  
 245 250 255  
 Glu Arg Arg Val Pro Phe Ser Leu Leu Arg Gly Pro Ser Trp Asp Pro  
 260 265 270  
 Phe Arg Asp Trp Tyr Pro His Ser Arg Leu Phe Asp Gln Ala Phe Gly  
 275 280 285  
 Leu Pro Arg Leu Pro Glu Glu Trp Ser Gln Trp Leu Gly Gly Ser Ser  
 290 295 300  
 Trp Pro Gly Tyr Val Arg Pro Leu Pro Pro Ala Ala Ile Glu Ser Pro  
 305 310 315 320  
 Ala Val Ala Ala Pro Ala Tyr Ser Arg Ala Leu Ser Arg Gln Leu Ser  
 325 330 335  
 Ser Gly Val Ser Glu Ile Arg His Thr Ala Asp Arg Trp Arg Val Ser  
 340 345 350  
 Leu Asp Val Asn His Phe Ala Pro Asp Glu Leu Thr Val Lys Thr Lys  
 355 360 365  
 Asp Gly Val Val Glu Ile Thr Gly Lys His Glu Glu Arg Gln Asp Glu  
 370 375 380  
 His Gly Tyr Ile Ser Arg Cys Phe Thr Arg Lys Tyr Thr Leu Pro Pro  
 385 390 395 400  
 Gly Val Asp Pro Thr Gln Val Ser Ser Ser Leu Ser Pro Glu Gly Thr  
 405 410 415  
 Leu Thr Val Glu Ala Pro Met Pro Lys Leu Ala Thr Gln Ser Asn Glu  
 420 425 430  
 Ile Thr Ile Pro Val Thr Phe Glu Ser Arg Ala Gln Leu Gly Gly Pro  
 435 440 445  
 Glu Ala Ala Lys Ser Asp Glu Thr Ala Ala Lys  
 450 455

&lt;210&gt; 171

&lt;211&gt; 2823

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: GFP-HSP70

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(2823)

&lt;400&gt; 171

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5					10					15		
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
		35					40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75					80		
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85					90						95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130					135					140					
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
			165					170						175		
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180					185					190			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624

Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu		
		195					200					205					
agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe		
	210					215				220							
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag	tcc	720	
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Ser		
	225				230				235						240		
gga	atg	tcg	gtg	gtg	ggc	ata	gac	ctg	ggc	ttc	cag	agc	tgc	tac	gtc	768	
Gly	Met	Ser	Val	Val	Gly	Ile	Asp	Leu	Gly	Phe	Gln	Ser	Cys	Tyr	Val		
				245					250					255			
gct	gtg	gcc	cgc	gcc	ggc	ggc	atc	gag	act	atc	gct	aat	gag	tat	agc	816	
Ala	Val	Ala	Arg	Ala	Gly	Gly	Ile	Glu	Thr	Ile	Ala	Asn	Glu	Tyr	Ser		
			260					265					270				
gac	cgc	tgc	acg	ccg	gct	tgc	att	tct	ttt	ggc	cct	aag	aat	cgt	tca	864	
Asp	Arg	Cys	Thr	Pro	Ala	Cys	Ile	Ser	Phe	Gly	Pro	Lys	Asn	Arg	Ser		
		275					280					285					
att	gga	gca	gca	gct	aaa	agc	cag	gta	att	tct	aat	gca	aag	aac	aca	912	
Ile	Gly	Ala	Ala	Ala	Lys	Ser	Gln	Val	Ile	Ser	Asn	Ala	Lys	Asn	Thr		
	290					295					300						
gtc	caa	gga	ttt	aaa	aga	ttc	cat	ggc	cga	gca	ttc	tct	gat	cca	ttt	960	
Val	Gln	Gly	Phe	Lys	Arg	Phe	His	Gly	Arg	Ala	Phe	Ser	Asp	Pro	Phe		
	305				310				315						320		
gtg	gag	gca	gaa	aaa	tct	aac	ctt	gca	tat	gat	att	gtg	cag	tgg	cct	1008	
Val	Glu	Ala	Glu	Lys	Ser	Asn	Leu	Ala	Tyr	Asp	Ile	Val	Gln	Trp	Pro		
				325					330					335			
aca	gga	tta	aca	ggt	ata	aag	gtg	aca	tat	atg	gag	gaa	gag	cga	aat	1056	
Thr	Gly	Leu	Thr	Gly	Ile	Lys	Val	Thr	Tyr	Met	Glu	Glu	Glu	Arg	Asn		
			340					345					350				
ttt	acc	act	gag	caa	gtg	act	gcc	atg	ctt	ttg	tcc	aaa	ctg	aag	gag	1104	
Phe	Thr	Thr	Glu	Gln	Val	Thr	Ala	Met	Leu	Leu	Ser	Lys	Leu	Lys	Glu		
		355					360					365					
aca	gcc	gaa	agt	gtt	ctt	aag	aag	cct	gta	gtt	gac	tgt	gtt	gtt	tcg	1152	
Thr	Ala	Glu	Ser	Val	Leu	Lys	Lys	Pro	Val	Val	Asp	Cys	Val	Val	Ser		
		370				375					380						
gtt	cct	tgt	ttc	tat	act	gat	gca	gaa	aga	cga	tca	gtg	atg	gat	gca	1200	
Val	Pro	Cys	Phe	Tyr	Thr	Asp	Ala	Glu	Arg	Arg	Ser	Val	Met	Asp	Ala		
	385				390				395						400		
aca	cag	att	gct	ggt	ctt	aat	tgc	ttg	cga	tta	atg	aat	gaa	acc	act	1248	
Thr	Gln	Ile	Ala	Gly	Leu	Asn	Cys	Leu	Arg	Leu	Met	Asn	Glu	Thr	Thr		
				405				410					415				
gca	gtt	gct	ctt	gca	tat	gga	atc	tat	aag	cag	gat	ctt	cct	cgc	tta	1296	
Ala	Val	Ala	Leu	Ala	Tyr	Gly	Ile	Tyr	Lys	Gln	Asp	Leu	Pro	Arg	Leu		

420						425						430						
gaa	gag	aaa	cca	aga	aat	gta	gtt	ttt	gta	gac	atg	ggc	cac	tct	gct	1344		
Glu	Glu	Lys	Pro	Arg	Asn	Val	Val	Phe	Val	Asp	Met	Gly	His	Ser	Ala			
435						440						445						
tat	caa	gtt	tct	gta	tgt	gca	ttt	aat	aga	gga	aaa	ctg	aaa	gtt	ctg	1392		
Tyr	Gln	Val	Ser	Val	Cys	Ala	Phe	Asn	Arg	Gly	Lys	Leu	Lys	Val	Leu			
450						455						460						
gcc	act	gca	ttt	gac	acg	aca	ttg	gga	ggg	aga	aaa	ttt	gat	gaa	gtg	1440		
Ala	Thr	Ala	Phe	Asp	Thr	Thr	Leu	Gly	Gly	Arg	Lys	Phe	Asp	Glu	Val			
465						470						475			480			
tta	gta	aat	cac	ttc	tgt	gaa	gaa	ttt	ggg	aag	aaa	tac	aag	cta	gac	1488		
Leu	Val	Asn	His	Phe	Cys	Glu	Glu	Phe	Gly	Lys	Lys	Tyr	Lys	Leu	Asp			
			485						490						495			
att	aag	tcc	aaa	atc	cgt	gca	tta	tta	cga	ctc	tct	cag	gag	tgt	gag	1536		
Ile	Lys	Ser	Lys	Ile	Arg	Ala	Leu	Leu	Arg	Leu	Ser	Gln	Glu	Cys	Glu			
			500						505						510			
aaa	ctc	aag	aaa	ttg	atg	agt	gca	aat	gct	tca	gat	ctc	cct	ttg	agc	1584		
Lys	Leu	Lys	Lys	Leu	Met	Ser	Ala	Asn	Ala	Ser	Asp	Leu	Pro	Leu	Ser			
			515						520						525			
att	gaa	tgt	ttt	atg	aat	gat	gtt	gat	gta	tct	gga	act	atg	aat	aga	1632		
Ile	Glu	Cys	Phe	Met	Asn	Asp	Val	Asp	Val	Ser	Gly	Thr	Met	Asn	Arg			
			530						535						540			
ggc	aaa	ttt	ctg	gag	atg	tgc	aat	gat	ctc	tta	gct	aga	gtg	gag	cca	1680		
Gly	Lys	Phe	Leu	Glu	Met	Cys	Asn	Asp	Leu	Leu	Ala	Arg	Val	Glu	Pro			
545						550						555			560			
cca	ctt	cgt	agt	gtt	ttg	gaa	caa	acc	aag	tta	aag	aaa	gaa	gat	att	1728		
Pro	Leu	Arg	Ser	Val	Leu	Glu	Gln	Thr	Lys	Leu	Lys	Lys	Glu	Asp	Ile			
			565						570						575			
tat	gca	gtg	gag	ata	gtt	ggg	ggg	gct	aca	cga	atc	cct	gcg	gta	aaa	1776		
Tyr	Ala	Val	Glu	Ile	Val	Gly	Gly	Ala	Thr	Arg	Ile	Pro	Ala	Val	Lys			
			580						585						590			
gag	aag	atc	agc	aaa	ttt	ttc	ggg	aaa	gaa	ctt	agt	aca	aca	tta	aat	1824		
Glu	Lys	Ile	Ser	Lys	Phe	Phe	Gly	Lys	Glu	Leu	Ser	Thr	Thr	Leu	Asn			
			595			600						605						
gct	gat	gaa	gct	gtc	act	cga	ggc	tgt	gca	ttg	cag	tgt	gcc	atc	tta	1872		
Ala	Asp	Glu	Ala	Val	Thr	Arg	Gly	Cys	Ala	Leu	Gln	Cys	Ala	Ile	Leu			
610						615						620						
tcg	cct	gct	ttc	aaa	gtc	aga	gaa	ttt	tct	atc	act	gat	gta	gta	cca	1920		
Ser	Pro	Ala	Phe	Lys	Val	Arg	Glu	Phe	Ser	Ile	Thr	Asp	Val	Val	Pro			
625						630						635			640			
tat	cca	ata	tct	ctg	aga	tgg	aat	tct	cca	gct	gaa	gaa	ggg	tca	agt	1968		
Tyr	Pro	Ile	Ser	Leu	Arg	Trp	Asn	Ser	Pro	Ala	Glu	Glu	Gly	Ser	Ser			
			645						650						655			

gac tgt gaa gtc ttt tcc aaa aat cat gct gct cct ttc tct aaa gtt Asp Cys Glu Val Phe Ser Lys Asn His Ala Ala Pro Phe Ser Lys Val 660 665 670	2016
ctt aca ttt tat aga aag gaa cct ttc act ctt gag gcc tac tac agc Leu Thr Phe Tyr Arg Lys Glu Pro Phe Thr Leu Glu Ala Tyr Tyr Ser 675 680 685	2064
tct cct cag gat ttg ccc tat cca gat cct gct ata gct cag ttt tca Ser Pro Gln Asp Leu Pro Tyr Pro Asp Pro Ala Ile Ala Gln Phe Ser 690 695 700	2112
gtt cag aaa gtc act cct cag tct gat ggc tcc agt tca aaa gtg aaa Val Gln Lys Val Thr Pro Gln Ser Asp Gly Ser Ser Ser Lys Val Lys 705 710 715 720	2160
gtc aaa gtt cga gta aat gtc cat ggc att ttc agt gtg tcc agt gca Val Lys Val Arg Val Asn Val His Gly Ile Phe Ser Val Ser Ser Ala 725 730 735	2208
tct tta gtg gag gtt cac aag tct gag gaa aat gag gag cca atg gaa Ser Leu Val Glu Val His Lys Ser Glu Glu Asn Glu Glu Pro Met Glu 740 745 750	2256
aca gat cag aat gca aag gag gaa gag aag atg caa gtg gac cag gag Thr Asp Gln Asn Ala Lys Glu Glu Glu Lys Met Gln Val Asp Gln Glu 755 760 765	2304
gaa cca cat gtt gaa gag caa cag cag cag aca cca gca gaa aat aag Glu Pro His Val Glu Glu Gln Gln Gln Gln Thr Pro Ala Glu Asn Lys 770 775 780	2352
gca gag tct gaa gaa atg gag acc tct caa gct gga tcc aag gat aaa Ala Glu Ser Glu Glu Met Glu Thr Ser Gln Ala Gly Ser Lys Asp Lys 785 790 795 800	2400
aag atg gac caa cca ccc caa tgc caa gaa ggc aaa agt gaa gac cag Lys Met Asp Gln Pro Pro Gln Cys Gln Glu Gly Lys Ser Glu Asp Gln 805 810 815	2448
tac tgt gga cct gcc aat cga gaa tca gct ata tgg cag ata gac aga Tyr Cys Gly Pro Ala Asn Arg Glu Ser Ala Ile Trp Gln Ile Asp Arg 820 825 830	2496
gag atg ctc aac ttg tac att gaa aat gag ggt aag atg atc atg cag Glu Met Leu Asn Leu Tyr Ile Glu Asn Glu Gly Lys Met Ile Met Gln 835 840 845	2544
gat aaa ctg gag aag gag cgg aat gat gct aag aac gca gtg gag gaa Asp Lys Leu Glu Lys Glu Arg Asn Asp Ala Lys Asn Ala Val Glu Glu 850 855 860	2592
tat gtg tat gaa atg aga gac aag ctt agt ggt gaa tat gag aag ttt Tyr Val Tyr Glu Met Arg Asp Lys Leu Ser Gly Glu Tyr Glu Lys Phe 865 870 875 880	2640

gtg agt gaa gat gat cgt aac agt ttt act ttg aaa ctg gaa gat act 2688  
Val Ser Glu Asp Asp Arg Asn Ser Phe Thr Leu Lys Leu Glu Asp Thr  
885 890 895

gaa aat tgg ttg tat gag gat gga gaa gac cag cca aag caa gtt tat 2736  
Glu Asn Trp Leu Tyr Glu Asp Gly Glu Asp Gln Pro Lys Gln Val Tyr  
900 905 910

gtt gat aag ttg gct gaa tta aaa aat cta ggt caa cct att aag ata 2784  
Val Asp Lys Leu Ala Glu Leu Lys Asn Leu Gly Gln Pro Ile Lys Ile  
915 920 925

cgt ttc cag gaa tct gaa gaa cga cca aat tat ttg aag 2823  
Arg Phe Gln Glu Ser Glu Glu Arg Pro Asn Tyr Leu Lys  
930 935 940

&lt;210&gt; 172

&lt;211&gt; 941

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: GFP-HSP70

&lt;400&gt; 172

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
50 55 60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser



139

465		470		475		480
Leu Val Asn His Phe Cys Glu Glu Phe Gly Lys Lys Tyr Lys Leu Asp						
		485		490		495
Ile Lys Ser Lys Ile Arg Ala Leu Leu Arg Leu Ser Gln Glu Cys Glu						
		500		505		510
Lys Leu Lys Lys Leu Met Ser Ala Asn Ala Ser Asp Leu Pro Leu Ser						
		515		520		525
Ile Glu Cys Phe Met Asn Asp Val Asp Val Ser Gly Thr Met Asn Arg						
		530		535		540
Gly Lys Phe Leu Glu Met Cys Asn Asp Leu Leu Ala Arg Val Glu Pro						
		545		550		555
Pro Leu Arg Ser Val Leu Glu Gln Thr Lys Leu Lys Lys Glu Asp Ile						
		565		570		575
Tyr Ala Val Glu Ile Val Gly Gly Ala Thr Arg Ile Pro Ala Val Lys						
		580		585		590
Glu Lys Ile Ser Lys Phe Phe Gly Lys Glu Leu Ser Thr Thr Leu Asn						
		595		600		605
Ala Asp Glu Ala Val Thr Arg Gly Cys Ala Leu Gln Cys Ala Ile Leu						
		610		615		620
Ser Pro Ala Phe Lys Val Arg Glu Phe Ser Ile Thr Asp Val Val Pro						
		625		630		635
Tyr Pro Ile Ser Leu Arg Trp Asn Ser Pro Ala Glu Glu Gly Ser Ser						
		645		650		655
Asp Cys Glu Val Phe Ser Lys Asn His Ala Ala Pro Phe Ser Lys Val						
		660		665		670
Leu Thr Phe Tyr Arg Lys Glu Pro Phe Thr Leu Glu Ala Tyr Tyr Ser						
		675		680		685
Ser Pro Gln Asp Leu Pro Tyr Pro Asp Pro Ala Ile Ala Gln Phe Ser						
		690		695		700
Val Gln Lys Val Thr Pro Gln Ser Asp Gly Ser Ser Ser Lys Val Lys						
		705		710		715
Val Lys Val Arg Val Asn Val His Gly Ile Phe Ser Val Ser Ser Ala						
		725		730		735
Ser Leu Val Glu Val His Lys Ser Glu Glu Asn Glu Glu Pro Met Glu						
		740		745		750
Thr Asp Gln Asn Ala Lys Glu Glu Glu Lys Met Gln Val Asp Gln Glu						
		755		760		765
Glu Pro His Val Glu Glu Gln Gln Gln Gln Thr Pro Ala Glu Asn Lys						

770	775	780
Ala Glu Ser Glu Glu Met Glu Thr Ser Gln Ala Gly Ser Lys Asp Lys 785	790	795 800
Lys Met Asp Gln Pro Pro Gln Cys Gln Glu Gly Lys Ser Glu Asp Gln 805	810	815
Tyr Cys Gly Pro Ala Asn Arg Glu Ser Ala Ile Trp Gln Ile Asp Arg 820	825	830
Glu Met Leu Asn Leu Tyr Ile Glu Asn Glu Gly Lys Met Ile Met Gln 835	840	845
Asp Lys Leu Glu Lys Glu Arg Asn Asp Ala Lys Asn Ala Val Glu Glu 850	855	860
Tyr Val Tyr Glu Met Arg Asp Lys Leu Ser Gly Glu Tyr Glu Lys Phe 865	870	875 880
Val Ser Glu Asp Asp Arg Asn Ser Phe Thr Leu Lys Leu Glu Asp Thr 885	890	895
Glu Asn Trp Leu Tyr Glu Asp Gly Glu Asp Gln Pro Lys Gln Val Tyr 900	905	910
Val Asp Lys Leu Ala Glu Leu Lys Asn Leu Gly Gln Pro Ile Lys Ile 915	920	925
Arg Phe Gln Glu Ser Glu Glu Arg Pro Asn Tyr Leu Lys 930	935	940

&lt;210&gt; 173

&lt;211&gt; 2674

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: GFP-HSC70

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(2673)

&lt;400&gt; 173

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg	48
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	

tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	192
50 55 60	
ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	240
65 70 75 80	
cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	288
85 90 95	
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	336
100 105 110	
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	384
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	432
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	480
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	528
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	576
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	624
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	672
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	720
225 230 235 240	
gga ctc aga tct atg tcc aag gga cct gca gtt ggt att gat ctt ggc Gly Leu Arg Ser Met Ser Lys Gly Pro Ala Val Gly Ile Asp Leu Gly	768
245 250 255	
acc acc tac tct tgt gtg ggt gtt ttc cag cac gga aaa gtc gag ata Thr Thr Tyr Ser Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile	816
260 265 270	

att gcc aat gat cag gga aac cga acc act cca agc tat gtc gcc ttt	864
Ile Ala Asn Asp Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe	
275 280 285	
acg gac act gaa cgg ttg atc ggt gat gcc gca aag aat caa gtt gca	912
Thr Asp Thr Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala	
290 295 300	
atg aac ccc acc aac aca gtt ttt gat gcc aaa cgt ctg att gga cgc	960
Met Asn Pro Thr Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg	
305 310 315 320	
aga ttt gat gat gct gtt gtc cag tct gat atg aaa cat tgg ccc ttt	1008
Arg Phe Asp Asp Ala Val Val Gln Ser Asp Met Lys His Trp Pro Phe	
325 330 335	
atg gtg gtg aat gat gct ggc agg ccc aag gtc caa gta gaa tac aag	1056
Met Val Val Asn Asp Ala Gly Arg Pro Lys Val Gln Val Glu Tyr Lys	
340 345 350	
gga gag acc aaa agc ttc tat cca gag gag gtg tct tct atg gtt ctg	1104
Gly Glu Thr Lys Ser Phe Tyr Pro Glu Glu Val Ser Ser Met Val Leu	
355 360 365	
aca aag atg aag gaa att gca gaa gcc tac ctt ggg aag act gtt acc	1152
Thr Lys Met Lys Glu Ile Ala Glu Ala Tyr Leu Gly Lys Thr Val Thr	
370 375 380	
aat gct gtg gtc aca gtg cca gct tac ttt aat gac tct cag cgt cag	1200
Asn Ala Val Val Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln	
385 390 395 400	
gct acc aaa gat gct gga act att gct ggt ctc aat gta ctt aga att	1248
Ala Thr Lys Asp Ala Gly Thr Ile Ala Gly Leu Asn Val Leu Arg Ile	
405 410 415	
att aat gag cca act gct gct gct att gct tac ggc tta gac aaa aag	1296
Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys	
420 425 430	
gtt gga gca gaa aga aac gtg ctc atc ttt gac ctg gga ggt ggc act	1344
Val Gly Ala Glu Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr	
435 440 445	
ttt gat gtg tca atc ctc act att gag gat gga atc ttt gag gtc aag	1392
Phe Asp Val Ser Ile Leu Thr Ile Glu Asp Gly Ile Phe Glu Val Lys	
450 455 460	
tct aca gct gga gac acc cac ttg ggt gga gaa gat ttt gac aac cga	1440
Ser Thr Ala Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg	
465 470 475 480	
atg gtc aac cat ttt att gct gag ttt aag cgc aag cat aag aag gac	1488
Met Val Asn His Phe Ile Ala Glu Phe Lys Arg Lys His Lys Lys Asp	
485 490 495	
atc agt gag aac aag aga gct gta aga cgc ctc cgt act gct tgt gaa	1536

Ile	Ser	Glu	Asn	Lys	Arg	Ala	Val	Arg	Arg	Leu	Arg	Thr	Ala	Cys	Glu	
			500					505					510			
cgt	gct	aag	cgt	acc	ctc	tct	tcc	agc	acc	cag	gcc	agt	att	gag	atc	1584
Arg	Ala	Lys	Arg	Thr	Leu	Ser	Ser	Ser	Thr	Gln	Ala	Ser	Ile	Glu	Ile	
		515					520					525				
gat	tct	ctc	tat	gaa	gga	atc	gac	ttc	tat	acc	tcc	att	acc	cgt	gcc	1632
Asp	Ser	Leu	Tyr	Glu	Gly	Ile	Asp	Phe	Tyr	Thr	Ser	Ile	Thr	Arg	Ala	
		530				535					540					
cga	ttt	gaa	gaa	ctg	aat	gct	gac	ctg	ttc	cgt	ggc	acc	ctg	gac	cca	1680
Arg	Phe	Glu	Glu	Leu	Asn	Ala	Asp	Leu	Phe	Arg	Gly	Thr	Leu	Asp	Pro	
545					550					555					560	
gta	gag	aaa	gcc	ctt	cga	gat	gcc	aaa	cta	gac	aag	tca	cag	att	cat	1728
Val	Glu	Lys	Ala	Leu	Arg	Asp	Ala	Lys	Leu	Asp	Lys	Ser	Gln	Ile	His	
				565					570					575		
gat	att	gtc	ctg	gtt	ggc	ggc	tct	act	cgt	atc	ccc	aag	att	cag	aag	1776
Asp	Ile	Val	Leu	Val	Gly	Gly	Ser	Thr	Arg	Ile	Pro	Lys	Ile	Gln	Lys	
			580					585					590			
ctt	ctc	caa	gac	ttc	ttc	aat	gga	aaa	gaa	ctg	aat	aag	agc	atc	aac	1824
Leu	Leu	Gln	Asp	Phe	Phe	Asn	Gly	Lys	Glu	Leu	Asn	Lys	Ser	Ile	Asn	
		595					600					605				
cct	gat	gaa	gct	gtt	gct	tat	ggc	gca	gct	gtc	cag	gca	gcc	atc	ttg	1872
Pro	Asp	Glu	Ala	Val	Ala	Tyr	Gly	Ala	Ala	Val	Gln	Ala	Ala	Ile	Leu	
		610				615					620					
tct	gga	gac	aag	tct	gag	aat	gtt	caa	gat	ttg	ctg	ctc	ttg	gat	gtc	1920
Ser	Gly	Asp	Lys	Ser	Glu	Asn	Val	Gln	Asp	Leu	Leu	Leu	Leu	Asp	Val	
625					630					635					640	
act	cct	ctt	tcc	ctt	ggc	att	gaa	act	gct	ggc	gga	gtc	atg	act	gtc	1968
Thr	Pro	Leu	Ser	Leu	Gly	Ile	Glu	Thr	Ala	Gly	Gly	Val	Met	Thr	Val	
				645				650						655		
ctc	atc	aag	cgt	aat	acc	acc	att	cct	acc	aag	cag	aca	cag	acc	ttc	2016
Leu	Ile	Lys	Arg	Asn	Thr	Thr	Ile	Pro	Thr	Lys	Gln	Thr	Gln	Thr	Phe	
			660					665					670			
act	acc	tat	tct	gac	aac	cag	cct	ggc	gtg	ctt	att	cag	gtt	tat	gaa	2064
Thr	Thr	Tyr	Ser	Asp	Asn	Gln	Pro	Gly	Val	Leu	Ile	Gln	Val	Tyr	Glu	
		675					680					685				
ggc	gag	cgt	gcc	atg	aca	aag	gat	aac	aac	ctg	ctt	ggc	aag	ttt	gaa	2112
Gly	Glu	Arg	Ala	Met	Thr	Lys	Asp	Asn	Asn	Leu	Leu	Gly	Lys	Phe	Glu	
		690				695					700					
ctc	aca	ggc	ata	cct	cct	gca	ccc	cga	ggc	gtt	cct	cag	att	gaa	gtc	2160
Leu	Thr	Gly	Ile	Pro	Pro	Ala	Pro	Arg	Gly	Val	Pro	Gln	Ile	Glu	Val	
705					710					715					720	
act	ttt	gac	att	gat	gcc	aat	ggc	ata	ctc	aat	gtc	tct	gct	gtg	gac	2208
Thr	Phe	Asp	Ile	Asp	Ala	Asn	Gly	Ile	Leu	Asn	Val	Ser	Ala	Val	Asp	

	725		730		735	
aag agt acg gga aaa gag aac aag att act atc act aat gac aag ggc						2256
Lys Ser Thr Gly Lys Glu Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly						
	740		745		750	
cgt ttg agc aag gaa gac att gaa cgt atg gtc cag gaa gct gag aag						2304
Arg Leu Ser Lys Glu Asp Ile Glu Arg Met Val Gln Glu Ala Glu Lys						
	755		760		765	
tac aaa gct gaa gat gag aag cag agg gac aag gtg tca tcc aag aat						2352
Tyr Lys Ala Glu Asp Glu Lys Gln Arg Asp Lys Val Ser Ser Lys Asn						
	770		775		780	
tca ctt gag tcc tat gcc ttc aac atg aaa gca act gtt gaa gat gag						2400
Ser Leu Glu Ser Tyr Ala Phe Asn Met Lys Ala Thr Val Glu Asp Glu						
	785		790		795	800
aaa ctt caa ggc aag att aac gat gag gac aaa cag aag att ctg gac						2448
Lys Leu Gln Gly Lys Ile Asn Asp Glu Asp Lys Gln Lys Ile Leu Asp						
	805		810		815	
aag tgt aat gaa att atc aac tgg ctt gat aag aat cag act gct gag						2496
Lys Cys Asn Glu Ile Ile Asn Trp Leu Asp Lys Asn Gln Thr Ala Glu						
	820		825		830	
aag gaa gaa ttt gaa cat caa cag aaa gag ctg gag aaa gtt tgc aac						2544
Lys Glu Glu Phe Glu His Gln Gln Lys Glu Leu Glu Lys Val Cys Asn						
	835		840		845	
ccc atc atc acc aag ctg tac cag agt gca gga ggc atg cca gga gga						2592
Pro Ile Ile Thr Lys Leu Tyr Gln Ser Ala Gly Gly Met Pro Gly Gly						
	850		855		860	
atg cct ggg gga ttt cct ggt ggt gga gct cct ccc tct ggt ggt gct						2640
Met Pro Gly Gly Phe Pro Gly Gly Gly Ala Pro Pro Ser Gly Gly Ala						
	865		870		875	880
tcc tca ggg ccc acc att gaa gag gtt gat taa g						2674
Ser Ser Gly Pro Thr Ile Glu Glu Val Asp						
	885		890			

&lt;210&gt; 174

&lt;211&gt; 890

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: GFP-HSC70

&lt;400&gt; 174

Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu
1				5				10					15		

Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly
			20					25					30		

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60  
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240  
 Gly Leu Arg Ser Met Ser Lys Gly Pro Ala Val Gly Ile Asp Leu Gly  
 245 250 255  
 Thr Thr Tyr Ser Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile  
 260 265 270  
 Ile Ala Asn Asp Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe  
 275 280 285  
 Thr Asp Thr Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala  
 290 295 300  
 Met Asn Pro Thr Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg  
 305 310 315 320  
 Arg Phe Asp Asp Ala Val Val Gln Ser Asp Met Lys His Trp Pro Phe  
 325 330 335



Met Val Val Asn Asp Ala Gly Arg Pro Lys Val Gln Val Glu Tyr Lys  
 340 345 350  
 Gly Glu Thr Lys Ser Phe Tyr Pro Glu Glu Val Ser Ser Met Val Leu  
 355 360 365  
 Thr Lys Met Lys Glu Ile Ala Glu Ala Tyr Leu Gly Lys Thr Val Thr  
 370 375 380  
 Asn Ala Val Val Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln  
 385 390 395 400  
 Ala Thr Lys Asp Ala Gly Thr Ile Ala Gly Leu Asn Val Leu Arg Ile  
 405 410 415  
 Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys  
 420 425 430  
 Val Gly Ala Glu Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr  
 435 440 445  
 Phe Asp Val Ser Ile Leu Thr Ile Glu Asp Gly Ile Phe Glu Val Lys  
 450 455 460  
 Ser Thr Ala Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg  
 465 470 475 480  
 Met Val Asn His Phe Ile Ala Glu Phe Lys Arg Lys His Lys Lys Asp  
 485 490 495  
 Ile Ser Glu Asn Lys Arg Ala Val Arg Arg Leu Arg Thr Ala Cys Glu  
 500 505 510  
 Arg Ala Lys Arg Thr Leu Ser Ser Ser Thr Gln Ala Ser Ile Glu Ile  
 515 520 525  
 Asp Ser Leu Tyr Glu Gly Ile Asp Phe Tyr Thr Ser Ile Thr Arg Ala  
 530 535 540  
 Arg Phe Glu Glu Leu Asn Ala Asp Leu Phe Arg Gly Thr Leu Asp Pro  
 545 550 555 560  
 Val Glu Lys Ala Leu Arg Asp Ala Lys Leu Asp Lys Ser Gln Ile His  
 565 570 575  
 Asp Ile Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys Ile Gln Lys  
 580 585 590  
 Leu Leu Gln Asp Phe Phe Asn Gly Lys Glu Leu Asn Lys Ser Ile Asn  
 595 600 605  
 Pro Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Ile Leu  
 610 615 620  
 Ser Gly Asp Lys Ser Glu Asn Val Gln Asp Leu Leu Leu Leu Asp Val  
 625 630 635 640

Thr Pro Leu Ser Leu Gly Ile Glu Thr Ala Gly Gly Val Met Thr Val  
 645 650 655  
 Leu Ile Lys Arg Asn Thr Thr Ile Pro Thr Lys Gln Thr Gln Thr Phe  
 660 665 670  
 Thr Thr Tyr Ser Asp Asn Gln Pro Gly Val Leu Ile Gln Val Tyr Glu  
 675 680 685  
 Gly Glu Arg Ala Met Thr Lys Asp Asn Asn Leu Leu Gly Lys Phe Glu  
 690 695 700  
 Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu Val  
 705 710 715 720  
 Thr Phe Asp Ile Asp Ala Asn Gly Ile Leu Asn Val Ser Ala Val Asp  
 725 730 735  
 Lys Ser Thr Gly Lys Glu Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly  
 740 745 750  
 Arg Leu Ser Lys Glu Asp Ile Glu Arg Met Val Gln Glu Ala Glu Lys  
 755 760 765  
 Tyr Lys Ala Glu Asp Glu Lys Gln Arg Asp Lys Val Ser Ser Lys Asn  
 770 775 780  
 Ser Leu Glu Ser Tyr Ala Phe Asn Met Lys Ala Thr Val Glu Asp Glu  
 785 790 795 800  
 Lys Leu Gln Gly Lys Ile Asn Asp Glu Asp Lys Gln Lys Ile Leu Asp  
 805 810 815  
 Lys Cys Asn Glu Ile Ile Asn Trp Leu Asp Lys Asn Gln Thr Ala Glu  
 820 825 830  
 Lys Glu Glu Phe Glu His Gln Gln Lys Glu Leu Glu Lys Val Cys Asn  
 835 840 845  
 Pro Ile Ile Thr Lys Leu Tyr Gln Ser Ala Gly Gly Met Pro Gly Gly  
 850 855 860  
 Met Pro Gly Gly Phe Pro Gly Gly Gly Ala Pro Pro Ser Gly Gly Ala  
 865 870 875 880  
 Ser Ser Gly Pro Thr Ile Glu Glu Val Asp  
 885 890

&lt;210&gt; 175

&lt;211&gt; 2458

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: GFP-HSF1

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(2349)

&lt;400&gt; 175

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5				10					15			
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
			35				40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75						80	
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
				85					90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130					135					140					
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
				165				170					175			
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180					185					190			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	
		195				200						205				

agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe 210 215 220	672
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser 225 230 235 240	720
gga ctc aga tct cga gct caa gct tcg aat tct gca gtc gag atg gat Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Ala Val Glu Met Asp 245 250 255	768
ctg ccc gtg ggc ccc ggc gcg gcg ggg ccc agc aac gtc ccg gcc ttc Leu Pro Val Gly Pro Gly Ala Ala Gly Pro Ser Asn Val Pro Ala Phe 260 265 270	816
ctg acc aag ctg tgg acc ctc gtg agc gac ccg gac acc gac gcg ctc Leu Thr Lys Leu Trp Thr Leu Val Ser Asp Pro Asp Thr Asp Ala Leu 275 280 285	864
atc tgc tgg agc ccg agc ggg aac agc ttc cac gtg ttc gac cag ggc Ile Cys Trp Ser Pro Ser Gly Asn Ser Phe His Val Phe Asp Gln Gly 290 295 300	912
cag ttt gcc aag gag gtg ctg ccc aag tac ttc aag cac aac aac atg Gln Phe Ala Lys Glu Val Leu Pro Lys Tyr Phe Lys His Asn Asn Met 305 310 315 320	960
gcc agc ttc gtg cgg cag ctc aac atg tat ggc ttc cgg aaa gtg gtc Ala Ser Phe Val Arg Gln Leu Asn Met Tyr Gly Phe Arg Lys Val Val 325 330 335	1008
cac atc gag cag ggc ggc ctg gtc aag cca gag aga gac gac acg gag His Ile Glu Gln Gly Gly Leu Val Lys Pro Glu Arg Asp Asp Thr Glu 340 345 350	1056
ttc cag cac cca tgc ttc ctg cgt ggc cag gag cag ctc ctt gag aac Phe Gln His Pro Cys Phe Leu Arg Gly Gln Glu Gln Leu Leu Glu Asn 355 360 365	1104
atc aag agg aaa gtg acc agt gtg tcc acc ctg aag agt gaa gac ata Ile Lys Arg Lys Val Thr Ser Val Ser Thr Leu Lys Ser Glu Asp Ile 370 375 380	1152
aag atc cgc cag gac agc gtc acc aag ctg ctg acg gac gtg cag ctg Lys Ile Arg Gln Asp Ser Val Thr Lys Leu Leu Thr Asp Val Gln Leu 385 390 395 400	1200
atg aag ggg aag cag gag tgc atg gac tcc aag ctc ctg gcc atg aag Met Lys Gly Lys Gln Glu Cys Met Asp Ser Lys Leu Leu Ala Met Lys 405 410 415	1248
cat gag aat gag gct ctg tgg cgg gag gtg gcc agc ctt cgg cag aag His Glu Asn Glu Ala Leu Trp Arg Glu Val Ala Ser Leu Arg Gln Lys 420 425 430	1296

cat gcc cag caa cag aaa gtc gtc aac aag ctc att cag ttc ctg atc His Ala Gln Gln Gln Lys Val Val Asn Lys Leu Ile Gln Phe Leu Ile 435 440 445	1344
tca ctg gtg cag tca aac cgg atc ctg ggg gtg aag aga aag atc ccc Ser Leu Val Gln Ser Asn Arg Ile Leu Gly Val Lys Arg Lys Ile Pro 450 455 460	1392
ctg atg ctg aac gac agt ggc tca gca cat tcc atg ccc aag tat agc Leu Met Leu Asn Asp Ser Gly Ser Ala His Ser Met Pro Lys Tyr Ser 465 470 475 480	1440
cgg cag ttc tcc ctg gag cac gtc cac ggc tcg ggc ccc tac tcg gcc Arg Gln Phe Ser Leu Glu His Val His Gly Ser Gly Pro Tyr Ser Ala 485 490 495	1488
ccc tcc cca gcc tac agc agc tcc agc ctc tac gcc cct gat gct gtg Pro Ser Pro Ala Tyr Ser Ser Ser Ser Leu Tyr Ala Pro Asp Ala Val 500 505 510	1536
gcc agc tct gga ccc atc atc tcc gac atc acc gag ctg gct cct gcc Ala Ser Ser Gly Pro Ile Ile Ser Asp Ile Thr Glu Leu Ala Pro Ala 515 520 525	1584
agc ccc atg gcc tcc ccc ggc ggg agc ata gac gag agg ccc cta tcc Ser Pro Met Ala Ser Pro Gly Gly Ser Ile Asp Glu Arg Pro Leu Ser 530 535 540	1632
agc agc ccc ctg gtg cgt gtc aag gag gag ccc ccc agc ccg cct cag Ser Ser Pro Leu Val Arg Val Lys Glu Glu Pro Pro Ser Pro Pro Gln 545 550 555 560	1680
agc ccc cgg gta gag gag gcg agt ccc ggg cgc cca tct tcc gtg gac Ser Pro Arg Val Glu Glu Ala Ser Pro Gly Arg Pro Ser Ser Val Asp 565 570 575	1728
acc ctc ttg tcc ccg acc gcc ctc att gac tcc atc ctg cgg gag agt Thr Leu Leu Ser Pro Thr Ala Leu Ile Asp Ser Ile Leu Arg Glu Ser 580 585 590	1776
gaa cct gcc ccc gcc tcc gtc aca gcc ctc acg gac gcc agg ggc cac Glu Pro Ala Pro Ala Ser Val Thr Ala Leu Thr Asp Ala Arg Gly His 595 600 605	1824
acg gac acc gag ggc cgg cct ccc tcc ccc ccg ccc acc tcc acc cct Thr Asp Thr Glu Gly Arg Pro Pro Ser Pro Pro Pro Thr Ser Thr Pro 610 615 620	1872
gaa aag tgc ctc agc gta gcc tgc ctg gac aag aat gag ctc agt gac Glu Lys Cys Leu Ser Val Ala Cys Leu Asp Lys Asn Glu Leu Ser Asp 625 630 635 640	1920
cac ttg gat gct atg gac tcc aac ctg gat aac ctg cag acc atg gac His Leu Asp Ala Met Asp Ser Asn Leu Asp Asn Leu Gln Thr Met Leu 645 650 655	1968
agc agc cac ggc ttc agc gtg gac acc agt gcc ctg ctg gac ctg ttc	2016

Ser Ser His Gly Phe Ser Val Asp Thr Ser Ala Leu Leu Asp Leu Phe  
 660 665 670  
 agc ccc tcg gtg acc gtg ccc gac atg agc ctg cct gac ctt gac agc 2064  
 Ser Pro Ser Val Thr Val Pro Asp Met Ser Leu Pro Asp Leu Asp Ser  
 675 680 685  
 agc ctg gcc agt atc caa gag ctc ctg tct ccc cag gag ccc ccc agg 2112  
 Ser Leu Ala Ser Ile Gln Glu Leu Leu Ser Pro Gln Glu Pro Pro Arg  
 690 695 700  
 cct ccc gag gca gag aac agc agc ccg gat tca ggg aag cag ctg gtg 2160  
 Pro Pro Glu Ala Glu Asn Ser Ser Pro Asp Ser Gly Lys Gln Leu Val  
 705 710 715 720  
 cac tac aca gcg cag ccg ctg ttc ctg ctg gac ccc ggc tcc gtg gac 2208  
 His Tyr Thr Ala Gln Pro Leu Phe Leu Leu Asp Pro Gly Ser Val Asp  
 725 730 735  
 acc ggg agc aac gac ctg ccg gtg ctg ttt gag ctg gga gag ggc tcc 2256  
 Thr Gly Ser Asn Asp Leu Pro Val Leu Phe Glu Leu Gly Glu Gly Ser  
 740 745 750  
 tac ttc tcc gaa ggg gac ggc ttc gcc gag gac ccc acc atc tcc ctg 2304  
 Tyr Phe Ser Glu Gly Asp Gly Phe Ala Glu Asp Pro Thr Ile Ser Leu  
 755 760 765  
 ctg aca ggc tcg gag cct ccc aaa gcc aag gac ccc act gtc tcc 2349  
 Leu Thr Gly Ser Glu Pro Pro Lys Ala Lys Asp Pro Thr Val Ser  
 770 775 780  
 tagaggcccc ggaggagctg ggccagccgc ccacccccac cccagtgca gggctggtct 2409  
 tggggaggga gggcagcctc gcggtcttgg gcaactggtgg gtcggccgg 2458

&lt;210&gt; 176

&lt;211&gt; 783

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: GFP-HSF1

&lt;400&gt; 176

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240  
 Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Ala Val Glu Met Asp  
 245 250 255  
 Leu Pro Val Gly Pro Gly Ala Ala Gly Pro Ser Asn Val Pro Ala Phe  
 260 265 270  
 Leu Thr Lys Leu Trp Thr Leu Val Ser Asp Pro Asp Thr Asp Ala Leu  
 275 280 285  
 Ile Cys Trp Ser Pro Ser Gly Asn Ser Phe His Val Phe Asp Gln Gly  
 290 295 300  
 Gln Phe Ala Lys Glu Val Leu Pro Lys Tyr Phe Lys His Asn Asn Met  
 305 310 315 320  
 Ala Ser Phe Val Arg Gln Leu Asn Met Tyr Gly Phe Arg Lys Val Val  
 325 330 335  
 His Ile Glu Gln Gly Gly Leu Val Lys Pro Glu Arg Asp Asp Thr Glu  
 340 345 350  
 Phe Gln His Pro Cys Phe Leu Arg Gly Gln Glu Gln Leu Leu Glu Asn  
 355 360 365

Ile Lys Arg Lys Val Thr Ser Val Ser Thr Leu Lys Ser Glu Asp Ile  
 370 375 380  
 Lys Ile Arg Gln Asp Ser Val Thr Lys Leu Leu Thr Asp Val Gln Leu  
 385 390 395 400  
 Met Lys Gly Lys Gln Glu Cys Met Asp Ser Lys Leu Leu Ala Met Lys  
 405 410 415  
 His Glu Asn Glu Ala Leu Trp Arg Glu Val Ala Ser Leu Arg Gln Lys  
 420 425 430  
 His Ala Gln Gln Gln Lys Val Val Asn Lys Leu Ile Gln Phe Leu Ile  
 435 440 445  
 Ser Leu Val Gln Ser Asn Arg Ile Leu Gly Val Lys Arg Lys Ile Pro  
 450 455 460  
 Leu Met Leu Asn Asp Ser Gly Ser Ala His Ser Met Pro Lys Tyr Ser  
 465 470 475 480  
 Arg Gln Phe Ser Leu Glu His Val His Gly Ser Gly Pro Tyr Ser Ala  
 485 490 495  
 Pro Ser Pro Ala Tyr Ser Ser Ser Ser Leu Tyr Ala Pro Asp Ala Val  
 500 505 510  
 Ala Ser Ser Gly Pro Ile Ile Ser Asp Ile Thr Glu Leu Ala Pro Ala  
 515 520 525  
 Ser Pro Met Ala Ser Pro Gly Gly Ser Ile Asp Glu Arg Pro Leu Ser  
 530 535 540  
 Ser Ser Pro Leu Val Arg Val Lys Glu Glu Pro Pro Ser Pro Pro Gln  
 545 550 555 560  
 Ser Pro Arg Val Glu Glu Ala Ser Pro Gly Arg Pro Ser Ser Val Asp  
 565 570 575  
 Thr Leu Leu Ser Pro Thr Ala Leu Ile Asp Ser Ile Leu Arg Glu Ser  
 580 585 590  
 Glu Pro Ala Pro Ala Ser Val Thr Ala Leu Thr Asp Ala Arg Gly His  
 595 600 605  
 Thr Asp Thr Glu Gly Arg Pro Pro Ser Pro Pro Pro Thr Ser Thr Pro  
 610 615 620  
 Glu Lys Cys Leu Ser Val Ala Cys Leu Asp Lys Asn Glu Leu Ser Asp  
 625 630 635 640  
 His Leu Asp Ala Met Asp Ser Asn Leu Asp Asn Leu Gln Thr Met Leu  
 645 650 655  
 Ser Ser His Gly Phe Ser Val Asp Thr Ser Ala Leu Leu Asp Leu Phe  
 660 665 670



Ser Pro Ser Val Thr Val Pro Asp Met Ser Leu Pro Asp Leu Asp Ser  
675 680 685

Ser Leu Ala Ser Ile Gln Glu Leu Leu Ser Pro Gln Glu Pro Pro Arg  
690 695 700

Pro Pro Glu Ala Glu Asn Ser Ser Pro Asp Ser Gly Lys Gln Leu Val  
705 710 715 720

His Tyr Thr Ala Gln Pro Leu Phe Leu Leu Asp Pro Gly Ser Val Asp  
725 730 735

Thr Gly Ser Asn Asp Leu Pro Val Leu Phe Glu Leu Gly Glu Gly Ser  
740 745 750

Tyr Phe Ser Glu Gly Asp Gly Phe Ala Glu Asp Pro Thr Ile Ser Leu  
755 760 765

Leu Thr Gly Ser Glu Pro Pro Lys Ala Lys Asp Pro Thr Val Ser  
770 775 780

<210> 177  
<211> 2416  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: GFP-NFKB

<220>  
<221> CDS  
<222> (1)..(2415)

<400> 177

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg	48
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag	240
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag	288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	

156

gtg cgc atc tcc ctg gtc acc aag gac cct cct cac cgg cct cac ccc	1008
Val Arg Ile Ser Leu Val Thr Lys Asp Pro Pro His Arg Pro His Pro	
325 330 335	
cac gag ctt gta gga aag gac tgc cgg gat ggc ttc tat gag gct gag	1056
His Glu Leu Val Gly Lys Asp Cys Arg Asp Gly Phe Tyr Glu Ala Glu	
340 345 350	
ctc tgc ccg gac cgc tgc atc cac agt ttc cag aac ctg gga atc cag	1104
Leu Cys Pro Asp Arg Cys Ile His Ser Phe Gln Asn Leu Gly Ile Gln	
355 360 365	
tgt gtg aag aag cgg gac ctg gag cag gct atc agt cag cgc atc cag	1152
Cys Val Lys Lys Arg Asp Leu Glu Gln Ala Ile Ser Gln Arg Ile Gln	
370 375 380	
acc aac aac aac ccc ttc caa gtt cct ata gaa gag cag cgt ggg gac	1200
Thr Asn Asn Asn Pro Phe Gln Val Pro Ile Glu Glu Gln Arg Gly Asp	
385 390 395 400	
tac gac ctg aat gct gtg cgg ctc tgc ttc cag gtg aca gtg cgg gac	1248
Tyr Asp Leu Asn Ala Val Arg Leu Cys Phe Gln Val Thr Val Arg Asp	
405 410 415	
cca tca ggc agg ccc ctc cgc ctg ccg cct gtc ctt tct cat ccc atc	1296
Pro Ser Gly Arg Pro Leu Arg Leu Pro Pro Val Leu Ser His Pro Ile	
420 425 430	
ttt gac aat cgt gcc ccc aac act gcc gag ctc aag atc tgc cga gtg	1344
Phe Asp Asn Arg Ala Pro Asn Thr Ala Glu Leu Lys Ile Cys Arg Val	
435 440 445	
aac cga aac tct ggc agc tgc ctc ggt ggg gat gag atc ttc cta ctg	1392
Asn Arg Asn Ser Gly Ser Cys Leu Gly Gly Asp Glu Ile Phe Leu Leu	
450 455 460	
tgt gac aag gtg cag aaa gag gac att gag gtg tat ttc acg gga cca	1440
Cys Asp Lys Val Gln Lys Glu Asp Ile Glu Val Tyr Phe Thr Gly Pro	
465 470 475 480	
ggc tgg gag gcc cga ggc tcc ttt tcg caa gct gat gtg cac cga caa	1488
Gly Trp Glu Ala Arg Gly Ser Phe Ser Gln Ala Asp Val His Arg Gln	
485 490 495	
gtg gcc att gtg ttc cgg acc cct ccc tac gca gac ccc agc ctg cag	1536
Val Ala Ile Val Phe Arg Thr Pro Pro Tyr Ala Asp Pro Ser Leu Gln	
500 505 510	
gct cct gtg cgt gtc tcc atg cag ctg cgg cgg cct tcc gac cgg gag	1584
Ala Pro Val Arg Val Ser Met Gln Leu Arg Arg Pro Ser Asp Arg Glu	
515 520 525	
ctc agt gag ccc atg gaa ttc cag tac ctg cca gat aca gac gat cgt	1632
Leu Ser Glu Pro Met Glu Phe Gln Tyr Leu Pro Asp Thr Asp Asp Arg	
530 535 540	

cac cgg att gag gag aaa cgt aaa agg aca tat gag acc ttc aag agc	1680
His Arg Ile Glu Glu Lys Arg Lys Arg Thr Tyr Glu Thr Phe Lys Ser	
545 550 555 560	
atc atg aag aag agt cct ttc agc gga ccc acc gac ccc cgg cct cca	1728
Ile Met Lys Lys Ser Pro Phe Ser Gly Pro Thr Asp Pro Arg Pro Pro	
565 570 575	
cct cga cgc att gct gtg cct tcc cgc agc tca gct tct gtc ccc aag	1776
Pro Arg Arg Ile Ala Val Pro Ser Arg Ser Ser Ala Ser Val Pro Lys	
580 585 590	
cca gca ccc cag ccc tat ccc ttt acg tca tcc ctg agc acc atc aac	1824
Pro Ala Pro Gln Pro Tyr Pro Phe Thr Ser Ser Leu Ser Thr Ile Asn	
595 600 605	
tat gat gag ttt ccc acc atg gtg ttt cct tct ggg cag atc agc cag	1872
Tyr Asp Glu Phe Pro Thr Met Val Phe Pro Ser Gly Gln Ile Ser Gln	
610 615 620	
gcc tcg gcc ttg gcc ccg gcc cct ccc caa gtc ctg ccc cag gct cca	1920
Ala Ser Ala Leu Ala Pro Ala Pro Pro Gln Val Leu Pro Gln Ala Pro	
625 630 635 640	
gcc cct gcc cct gct cca gcc atg gta tca gct ctg gcc cag gcc cca	1968
Ala Pro Ala Pro Ala Pro Ala Met Val Ser Ala Leu Ala Gln Ala Pro	
645 650 655	
gcc cct gtc cca gtc cta gcc cca ggc cct cct cag gct gtg gcc cca	2016
Ala Pro Val Pro Val Leu Ala Pro Gly Pro Pro Gln Ala Val Ala Pro	
660 665 670	
cct gcc ccc aag ccc acc cag gct ggg gaa gga acg ctg tca gag gcc	2064
Pro Ala Pro Lys Pro Thr Gln Ala Gly Glu Gly Thr Leu Ser Glu Ala	
675 680 685	
ctg ctg cag ctg cag ttt gat gat gaa gac ctg ggg gcc ttg ctt ggc	2112
Leu Leu Gln Leu Gln Phe Asp Asp Glu Asp Leu Gly Ala Leu Leu Gly	
690 695 700	
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Asn Ser Thr Asp Pro Ala Val Phe Thr Asp Leu Ala Ser Val Asp Asn	
705 710 715 720	
tcc gag ttt cag cag ctg ctg aac cag ggc ata cct gtg gcc ccc cac	2208
Ser Glu Phe Gln Gln Leu Leu Asn Gln Gly Ile Pro Val Ala Pro His	
725 730 735	
aca act gag ccc atg ctg atg gag tac cct gag gct ata act cgc cta	2256
Thr Thr Glu Pro Met Leu Met Glu Tyr Pro Glu Ala Ile Thr Arg Leu	
740 745 750	
gtg aca gcc cag agg ccc ccc gac cca gct cct gct cca ctg ggg gcc	2304
Val Thr Ala Gln Arg Pro Pro Asp Pro Ala Pro Ala Pro Leu Gly Ala	
755 760 765	
ccg ggg ctc ccc aat ggc ctc ctt tca gga gat gaa gac ttc tcc tcc	2352

Pro Gly Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser  
 770 775 780

att gcg gac atg gac ttc tca gcc ctg ctg agt cag atc agc tcc aag 2400  
 Ile Ala Asp Met Asp Phe Ser Ala Leu Leu Ser Gln Ile Ser Ser Lys  
 785 790 795 800

ggc gaa ttc gaa gct t 2416  
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<210> 178

<211> 805

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-NFKB

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Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240  
 Gly Leu Arg Ser Arg Asp Pro Pro Phe Met Asp Glu Leu Phe Pro Leu  
 245 250 255  
 Ile Phe Pro Ala Glu Pro Ala Gln Ala Ser Gly Pro Tyr Val Glu Ile  
 260 265 270  
 Ile Glu Gln Pro Lys Gln Arg Gly Met Arg Phe Arg Tyr Lys Cys Glu  
 275 280 285  
 Gly Arg Ser Ala Gly Ser Ile Pro Gly Glu Arg Ser Thr Asp Thr Thr  
 290 295 300  
 Lys Thr His Pro Thr Ile Lys Ile Asn Gly Tyr Thr Gly Pro Gly Thr  
 305 310 315 320  
 Val Arg Ile Ser Leu Val Thr Lys Asp Pro Pro His Arg Pro His Pro  
 325 330 335  
 His Glu Leu Val Gly Lys Asp Cys Arg Asp Gly Phe Tyr Glu Ala Glu  
 340 345 350  
 Leu Cys Pro Asp Arg Cys Ile His Ser Phe Gln Asn Leu Gly Ile Gln  
 355 360 365  
 Cys Val Lys Lys Arg Asp Leu Glu Gln Ala Ile Ser Gln Arg Ile Gln  
 370 375 380  
 Thr Asn Asn Asn Pro Phe Gln Val Pro Ile Glu Glu Gln Arg Gly Asp  
 385 390 395 400  
 Tyr Asp Leu Asn Ala Val Arg Leu Cys Phe Gln Val Thr Val Arg Asp  
 405 410 415  
 Pro Ser Gly Arg Pro Leu Arg Leu Pro Pro Val Leu Ser His Pro Ile  
 420 425 430  
 Phe Asp Asn Arg Ala Pro Asn Thr Ala Glu Leu Lys Ile Cys Arg Val  
 435 440 445  
 Asn Arg Asn Ser Gly Ser Cys Leu Gly Gly Asp Glu Ile Phe Leu Leu  
 450 455 460  
 Cys Asp Lys Val Gln Lys Glu Asp Ile Glu Val Tyr Phe Thr Gly Pro  
 465 470 475 480  
 Gly Trp Glu Ala Arg Gly Ser Phe Ser Gln Ala Asp Val His Arg Gln  
 485 490 495

Val Ala Ile Val Phe Arg Thr Pro Pro Tyr Ala Asp Pro Ser Leu Gln  
 500 505 510  
 Ala Pro Val Arg Val Ser Met Gln Leu Arg Arg Pro Ser Asp Arg Glu  
 515 520 525  
 Leu Ser Glu Pro Met Glu Phe Gln Tyr Leu Pro Asp Thr Asp Asp Arg  
 530 535 540  
 His Arg Ile Glu Glu Lys Arg Lys Arg Thr Tyr Glu Thr Phe Lys Ser  
 545 550 555 560  
 Ile Met Lys Lys Ser Pro Phe Ser Gly Pro Thr Asp Pro Arg Pro Pro  
 565 570 575  
 Pro Arg Arg Ile Ala Val Pro Ser Arg Ser Ser Ala Ser Val Pro Lys  
 580 585 590  
 Pro Ala Pro Gln Pro Tyr Pro Phe Thr Ser Ser Leu Ser Thr Ile Asn  
 595 600 605  
 Tyr Asp Glu Phe Pro Thr Met Val Phe Pro Ser Gly Gln Ile Ser Gln  
 610 615 620  
 Ala Ser Ala Leu Ala Pro Ala Pro Pro Gln Val Leu Pro Gln Ala Pro  
 625 630 635 640  
 Ala Pro Ala Pro Ala Pro Ala Met Val Ser Ala Leu Ala Gln Ala Pro  
 645 650 655  
 Ala Pro Val Pro Val Leu Ala Pro Gly Pro Pro Gln Ala Val Ala Pro  
 660 665 670  
 Pro Ala Pro Lys Pro Thr Gln Ala Gly Glu Gly Thr Leu Ser Glu Ala  
 675 680 685  
 Leu Leu Gln Leu Gln Phe Asp Asp Glu Asp Leu Gly Ala Leu Leu Gly  
 690 695 700  
 Asn Ser Thr Asp Pro Ala Val Phe Thr Asp Leu Ala Ser Val Asp Asn  
 705 710 715 720  
 Ser Glu Phe Gln Gln Leu Leu Asn Gln Gly Ile Pro Val Ala Pro His  
 725 730 735  
 Thr Thr Glu Pro Met Leu Met Glu Tyr Pro Glu Ala Ile Thr Arg Leu  
 740 745 750  
 Val Thr Ala Gln Arg Pro Pro Asp Pro Ala Pro Ala Pro Leu Gly Ala  
 755 760 765  
 Pro Gly Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser  
 770 775 780  
 Ile Ala Asp Met Asp Phe Ser Ala Leu Leu Ser Gln Ile Ser Ser Lys  
 785 790 795 800

Gly Glu Phe Glu Ala  
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<210> 179

<211> 1677

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-IKB

<220>

<221> CDS

<222> (1) .. (1674)

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cgc gac ggg ctg aag aag gag cgg cta ctg gac gac cgc cac gac agc	96
Arg Asp Gly Leu Lys Lys Glu Arg Leu Leu Asp Asp Arg His Asp Ser	
20 25 30	
ggc ctg gac tcc atg aaa gac gag gag tac gag cag atg gtc aag gag	144
Gly Leu Asp Ser Met Lys Asp Glu Glu Tyr Glu Gln Met Val Lys Glu	
35 40 45	
ctg cag gag atc cgc ctc gag ccg cag gag gtg ccg cgc ggc tcg gag	192
Leu Gln Glu Ile Arg Leu Glu Pro Gln Glu Val Pro Arg Gly Ser Glu	
50 55 60	
ccc tgg aag cag cag ctc acc gag gac ggg gac tcg ttc ctg cac ttg	240
Pro Trp Lys Gln Gln Leu Thr Glu Asp Gly Asp Ser Phe Leu His Leu	
65 70 75 80	
gcc atc atc cat gaa gaa aag gca ctg acc atg gaa gtg atc cgc cag	288
Ala Ile Ile His Glu Glu Lys Ala Leu Thr Met Glu Val Ile Arg Gln	
85 90 95	
gtg aag gga gac ctg gcc ttc ctc aac ctc cag aac aac ctg cag cag	336
Val Lys Gly Asp Leu Ala Phe Leu Asn Leu Gln Asn Asn Leu Gln Gln	
100 105 110	
act cca ctc cac ttg gct gtg atc acc aac cag cca gaa att gct gag	384
Thr Pro Leu His Leu Ala Val Ile Thr Asn Gln Pro Glu Ile Ala Glu	
115 120 125	
gca ctt ctg gga gct ggc tgt gat cct gag ctc cga gac ttt cga gga	432
Ala Leu Leu Gly Ala Gly Cys Asp Pro Glu Leu Arg Asp Phe Arg Gly	
130 135 140	
aat acc ccc cta cac ctt gcc tgt gag cag ggc tgc ctg gcc agc gtg	480
Asn Thr Pro Leu His Leu Ala Cys Glu Gln Gly Cys Leu Ala Ser Val	
145 150 155 160	



gga gtc ctg act cag tcc tgc acc acc ccg cac ctc cac tcc atc ttg	528
Gly Val Leu Thr Gln Ser Cys Thr Thr Pro His Leu His Ser Ile Leu	
165 170 175	
aag gct acc aac tac aat ggc cac acg tgt cta cac tta gcc tct atc	576
Lys Ala Thr Asn Tyr Asn Gly His Thr Cys Leu His Leu Ala Ser Ile	
180 185 190	
cat ggc tac ctg ggc atc gtg gag ctt ttg gtg tcc ttg ggt gct gat	624
His Gly Tyr Leu Gly Ile Val Glu Leu Leu Val Ser Leu Gly Ala Asp	
195 200 205	
gtc aat gct cag gag ccc tgt aat ggc cgg act gcc ctt cac ctc gca	672
Val Asn Ala Gln Glu Pro Cys Asn Gly Arg Thr Ala Leu His Leu Ala	
210 215 220	
gtg gac ctg caa aat cct gac ctg gtg tca ctc ctg ttg aag tgt ggg	720
Val Asp Leu Gln Asn Pro Asp Leu Val Ser Leu Leu Leu Lys Cys Gly	
225 230 235 240	
gct gat gtc aac aga gtt acc tac cag ggc tat tct ccc tac cag ctc	768
Ala Asp Val Asn Arg Val Thr Tyr Gln Gly Tyr Ser Pro Tyr Gln Leu	
245 250 255	
acc tgg ggc cgc cca agc acc cgg ata cag cag cag ctg ggc cag ctg	816
Thr Trp Gly Arg Pro Ser Thr Arg Ile Gln Gln Gln Leu Gly Gln Leu	
260 265 270	
aca cta gaa aac ctt cag atg ctg cca gag agt gag gat gag gag agc	864
Thr Leu Glu Asn Leu Gln Met Leu Pro Glu Ser Glu Asp Glu Glu Ser	
275 280 285	
tat gac aca gag tca gag ttc acg gag ttc aca gag gac gag ctg ccc	912
Tyr Asp Thr Glu Ser Glu Phe Thr Glu Phe Thr Glu Asp Glu Leu Pro	
290 295 300	
tat gat gac tgt gtg ttt gga ggc cag cgt ctg acg tta acc ggt atg	960
Tyr Asp Asp Cys Val Phe Gly Gly Gln Arg Leu Thr Leu Thr Gly Met	
305 310 315 320	
gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt gtt	1008
Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val	
325 330 335	
gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga gag	1056
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu	
340 345 350	
ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc tgc	1104
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys	
355 360 365	
act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act ctg	1152
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu	
370 375 380	
tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa cgg	1200

Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	Arg	
385					390					395					400	
cat	gac	ttt	ttc	aag	agt	gcc	atg	ccc	gaa	ggg	tat	gta	cag	gaa	agg	1248
His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	Arg	
				405					410					415		
acc	atc	ttc	ttc	aaa	gat	gac	ggc	aac	tac	aag	aca	cgt	gct	gaa	gtc	1296
Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	Val	
				420				425					430			
aag	ttt	gaa	ggg	gat	acc	ctt	gtt	aat	aga	atc	gag	tta	aaa	ggg	att	1344
Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile	
		435					440					445				
gac	ttc	aag	gaa	gat	ggc	aac	att	ctg	gga	cac	aaa	ttg	gaa	tac	aac	1392
Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn	
		450				455					460					
tat	aac	tca	cac	aat	gta	tac	atc	atg	gca	gac	aaa	caa	aag	aat	gga	1440
Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly	
465					470					475					480	
atc	aaa	gtg	aac	ttc	aag	acc	cgc	cac	aac	att	gaa	gat	gga	agc	gtt	1488
Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val	
				485					490					495		
caa	cta	gca	gac	cat	tat	caa	caa	aat	act	cca	att	ggc	gat	ggc	cct	1536
Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro	
			500					505					510			
gtc	ctt	tta	cca	gac	aac	cat	tac	ctg	tcc	aca	caa	tct	gcc	ctt	tcg	1584
Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser	
			515				520					525				
aaa	gat	ccc	aac	gaa	aag	aga	gac	cac	atg	gtc	ctt	ctt	gag	ttt	gta	1632
Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val	
		530				535					540					
aca	gct	gct	ggg	att	aca	cat	ggc	atg	gat	gaa	ctg	tac	aac	tag		1677
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&lt;210&gt; 180

&lt;211&gt; 558

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: GFP-IKB

&lt;400&gt; 180

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Arg	Asp	Gly	Leu	Lys	Lys	Glu	Arg	Leu	Leu	Asp	Asp	Arg	His	Asp	Ser
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

20					25					30						
Gly	Leu	Asp	Ser	Met	Lys	Asp	Glu	Glu	Tyr	Glu	Gln	Met	Val	Lys	Glu	
35					40					45						
Leu	Gln	Glu	Ile	Arg	Leu	Glu	Pro	Gln	Glu	Val	Pro	Arg	Gly	Ser	Glu	
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Pro	Trp	Lys	Gln	Gln	Leu	Thr	Glu	Asp	Gly	Asp	Ser	Phe	Leu	His	Leu	
65					70					75					80	
Ala	Ile	Ile	His	Glu	Gln	Lys	Ala	Leu	Thr	Met	Glu	Val	Ile	Arg	Gln	
85					90					95						
Val	Lys	Gly	Asp	Leu	Ala	Phe	Leu	Asn	Leu	Gln	Asn	Asn	Leu	Gln	Gln	
100					105					110						
Thr	Pro	Leu	His	Leu	Ala	Val	Ile	Thr	Asn	Gln	Pro	Glu	Ile	Ala	Glu	
115					120					125						
Ala	Leu	Leu	Gly	Ala	Gly	Cys	Asp	Pro	Glu	Leu	Arg	Asp	Phe	Arg	Gly	
130					135					140						
Asn	Thr	Pro	Leu	His	Leu	Ala	Cys	Glu	Gln	Gly	Cys	Leu	Ala	Ser	Val	
145					150					155					160	
Gly	Val	Leu	Thr	Gln	Ser	Cys	Thr	Thr	Pro	His	Leu	His	Ser	Ile	Leu	
165					170					175						
Lys	Ala	Thr	Asn	Tyr	Asn	Gly	His	Thr	Cys	Leu	His	Leu	Ala	Ser	Ile	
180					185					190						
His	Gly	Tyr	Leu	Gly	Ile	Val	Glu	Leu	Leu	Val	Ser	Leu	Gly	Ala	Asp	
195					200					205						
Val	Asn	Ala	Gln	Glu	Pro	Cys	Asn	Gly	Arg	Thr	Ala	Leu	His	Leu	Ala	
210					215					220						
Val	Asp	Leu	Gln	Asn	Pro	Asp	Leu	Val	Ser	Leu	Leu	Leu	Lys	Cys	Gly	
225					230					235					240	
Ala	Asp	Val	Asn	Arg	Val	Thr	Tyr	Gln	Gly	Tyr	Ser	Pro	Tyr	Gln	Leu	
245					250					255						
Thr	Trp	Gly	Arg	Pro	Ser	Thr	Arg	Ile	Gln	Gln	Gln	Leu	Gly	Gln	Leu	
260					265					270						
Thr	Leu	Glu	Asn	Leu	Gln	Met	Leu	Pro	Glu	Ser	Glu	Asp	Glu	Glu	Ser	
275					280					285						
Tyr	Asp	Thr	Glu	Ser	Glu	Phe	Thr	Glu	Phe	Thr	Glu	Asp	Glu	Leu	Pro	
290					295					300						
Tyr	Asp	Asp	Cys	Val	Phe	Gly	Gly	Gln	Arg	Leu	Thr	Leu	Thr	Gly	Met	
305					310					315					320	
Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	Val	

				325						330							335
Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	Glu		
			340					345					350				
Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	Cys		
		355					360					365					
Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	Leu		
	370					375						380					
Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	Arg		
385					390					395					400		
His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	Arg		
				405					410					415			
Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	Val		
			420					425					430				
Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile		
		435					440					445					
Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn		
	450					455					460						
Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly		
465					470					475					480		
Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val		
				485					490					495			
Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro		
			500					505					510				
Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser		
		515					520					525					
Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val		
	530					535					540						
Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Asn				
545					550					555							

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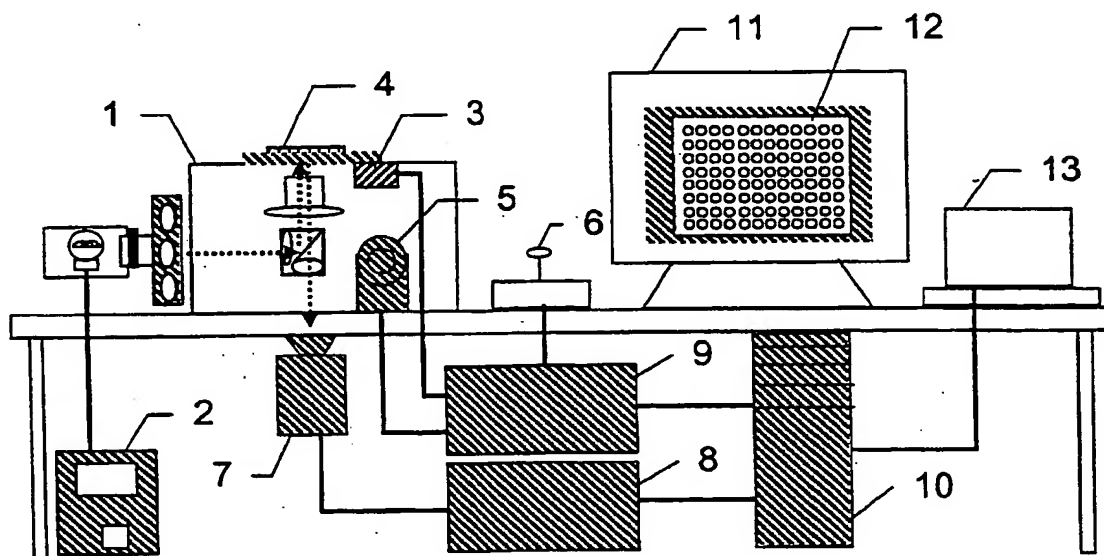
(72) Inventors; and

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(54) Title: A SYSTEM FOR CELL-BASED SCREENING



(57) Abstract: The present invention provides systems, methods, screens, reagents and kits for optical system analysis of cells to rapidly determine the distribution, environment, or activity of fluorescently labeled reporter molecules in cells for the purpose of screening large numbers of compounds for those that specifically affect particular biological functions.

WO 00/50872 A3

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/04794

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N15/14 G01N33/50 C12M1/34

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 45704 A (TULLIN SOEREN ;KASPER ALMHOLT (DK); NOVONORDISK AS (DK); SCUDDER K) 15 October 1998 (1998-10-15) abstract; figures 1,19	1,3,20
A	WO 97 38127 A (GEN HOSPITAL CORP ;GENETICS INST (US)) 16 October 1997 (1997-10-16) page 1, line 13 page 4, line 14 -page 5, line 17 page 12, line 9-15 --- -/-	1,3,20



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

27 October 2000

Date of mailing of the international search report

13.11.00

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/04794

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GIULIANO K A ET AL: "Fluorescent-protein biosensors: new tools for drug discovery" TRENDS IN BIOTECHNOLOGY, GB, ELSEVIER PUBLICATIONS, CAMBRIDGE, vol. 16, no. 3, 1 March 1998 (1998-03-01), pages 135-140, XP004108592 ISSN: 0167-7799 page 138, column 2, last paragraph page 139, column 2, paragraph 2</p>	1,3,20
A	<p>WO 98 38490 A (BIODX INC.; DUNLAY R TERRY (US); GOUGH ALBERT H (US); GIULIANO KENN) 3 September 1998 (1998-09-03) abstract page 7, line 27 -page 8, line 10</p>	1,3,20

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 00/04794

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17  
because they relate to subject matter not required to be searched by this Authority, namely:  
Rule 39.1(vi) PCT - Program for computers
2. ☒ Claims Nos.: 18, 19  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 18,19

there is lack of clarity as it is unclear if the instructions detailing the method are to be used to define the kit (not allowable as a method may not be used to define a product) or alternatively were to be regarded as a sheet of paper. F

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/04794

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9845704	A	15-10-1998	AU 6820998 A EP 0986753 A	30-10-1998 22-03-2000
WO 9738127	A	16-10-1997	EP 0904402 A JP 2000508174 T	31-03-1999 04-07-2000
WO 9838490	A	03-09-1998	US 5989835 A US 6103479 A AU 6667898 A EP 0983498 A JP 2000509827 T AU 3297197 A EP 0912892 A	23-11-1999 15-08-2000 18-09-1998 08-03-2000 02-08-2000 05-01-1998 06-05-1999

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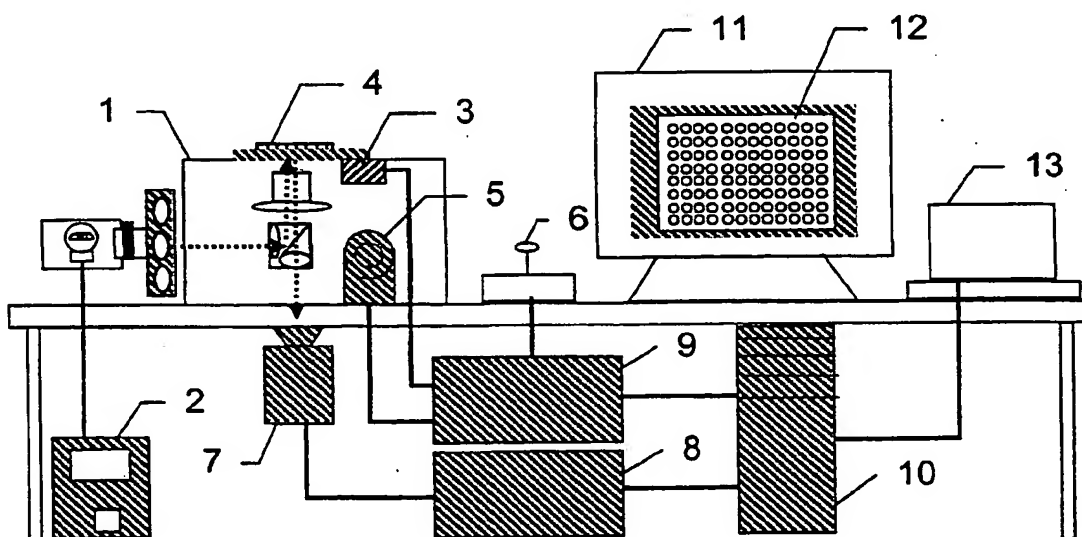
(74) Agent: **HARPER, David, S.**; McDonnell, Boehnen, Hul-  
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IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,  
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,  
RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA,  
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(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,  
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,  
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[Continued on next page]

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see PCT Gazette No. 46/2001 of 15 November 2001, Section II

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## A SYSTEM FOR CELL-BASED SCREENING

### 5 Cross Reference

This application claims priority to U.S. Provisional Applications for Patent Serial Nos. 60/122,152 (February 26, 1999), 60/123,399 (March 8, 1999), 09/352,141, (July 12, 1999), 60/151,797 (August 31, 1999), 60/168,408 (December 1, 1999); and is a continuation in part of 09/430,656 (October 29, 1999); 09/398,965 filed September 10 17, 1999 which is a continuation in part of Serial No. 09/031,271 filed February 27, 1998 which is a continuation in part of U.S. Application S/N 08/810983, filed on February 27, 1997.

### Field of The Invention

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This invention is in the field of fluorescence-based cell and molecular biochemical assays for drug discovery.

### Background of the Invention

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Drug discovery, as currently practiced in the art, is a long, multiple step process involving identification of specific disease targets, development of an assay based on a specific target, validation of the assay, optimization and automation of the assay to produce a screen, high throughput screening of compound libraries using the assay to 25 identify "hits", hit validation and hit compound optimization. The output of this process is a lead compound that goes into pre-clinical and, if validated, eventually into clinical trials. In this process, the screening phase is distinct from the assay development phases, and involves testing compound efficacy in living biological systems.

30

Historically, drug discovery is a slow and costly process, spanning numerous years and consuming hundreds of millions of dollars per drug created. Developments in the areas of genomics and high throughput screening have resulted in increased capacity and efficiency in the areas of target identification and volume of compounds

Historically, drug discovery is a slow and costly process, spanning numerous years and consuming hundreds of millions of dollars per drug created. Developments in the areas of genomics and high throughput screening have resulted in increased capacity and efficiency in the areas of target identification and volume of compounds screened. Significant advances in automated DNA sequencing, PCR application, positional cloning, hybridization arrays, and bioinformatics have greatly increased the number of genes (and gene fragments) encoding potential drug screening targets. However, the basic scheme for drug screening remains the same.

Validation of genomic targets as points for therapeutic intervention using the existing methods and protocols has become a bottleneck in the drug discovery process due to the slow, manual methods employed, such as *in vivo* functional models, functional analysis of recombinant proteins, and stable cell line expression of candidate genes. Primary DNA sequence data acquired through automated sequencing does not permit identification of gene function, but can provide information about common "motifs" and specific gene homology when compared to known sequence databases. Genomic methods such as subtraction hybridization and RADE (rapid amplification of differential expression) can be used to identify genes that are up or down regulated in a disease state model. However, identification and validation still proceed down the same pathway. Some proteomic methods use protein identification (global expression arrays, 2D electrophoresis, combinatorial libraries) in combination with reverse genetics to identify candidate genes of interest. Such putative "disease associated sequences" or DAS isolated as intact cDNA are a great advantage to these methods, but they are identified by the hundreds without providing any information regarding type, activity, and distribution of the encoded protein. Choosing a subset of DAS as drug screening targets is "random", and thus extremely inefficient, without functional data to provide a mechanistic link with disease. It is necessary, therefore, to provide new technologies to rapidly screen DAS to establish biological function, thereby improving target validation and candidate optimization in drug discovery.

There are three major avenues for improving early drug discovery productivity. First, there is a need for tools that provide increased information handling capability. Bioinformatics has blossomed with the rapid development of DNA sequencing systems and the evolution of the genomics database. Genomics is beginning to play a critical

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